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by

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Impacts of waterborne copper and silver on the early life stage (ELS) of zebrafish (*Danio rerio*): physiological, biochemical and molecular responses

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Abstract

Toxic metals are major pollutants of the aquatic environment and are able to cause survival impairment of the early life stage of the aquatic organisms. They can affect the osmoregulatory system and electrolyte balance in fishes as well as the expression of genes which are essential in the formation and development of the organs at the early embryonic stages of development. There are a lack of studies concerning the toxic effects of waterborne copper and silver on the osmoregulation, electrolytes balance and expression the genes which are responsible for the formation and development of heart and metal binding proteins in the early life stage of zebrafish. The current study aimed to assess the toxic effects of waterborne concentrations of copper as an essential trace element, and silver as a non-essential trace element, on biochemical processes and the molecular biology of the early life stages (ELS) of zebrafish. The first experiment of the current study (Chapter 3) aimed: 1. to determine the time of *nkx2.5* gene expression, a gene involved in cardiac development, relative to the time of embryonic development. 2. To assess the toxic concentration of the copper and most vulnerable and sensitive stage of the embryos < 1 hour post fertilization (hpf) exposed to the copper via water route. The result of the experiment showed that the expression of the *nkx2.5* gene reached a maximum at 16 hpf. The first 10 hpf of the embryonic development was the most vulnerable and critical stage of the developing embryos, and characterized by increased mortality as copper concentration increased, and delayed and decreased hatching success. Exposure of embryos for 72 hpf to a concentration of 500 µg L^{-1} Cu increased heart rate, whereas the exposure of the embryos at the blastula stage only, showed decreases in heart rate. The third part of the experiment evaluated the protective

effect of calcium as a major cation of water hardness on Cu toxicity. Embryos age < 2 hpf were exposed to copper (0, 100, 250, and 500 µg L^{-1}), with or without added calcium (40 mg L^{-1}). An increase in embryonic Cu accumulation was observed in live and dead embryos exposed to Cu, with and without added calcium. Calcium concentration increased with embryonic copper tissue concentration in dead embryos. Na^+ and K^+ concentrations were higher in live embryos compared to dead embryos, and a 4 fold decrease in Na^+K^+-ATPase activity was seen in live embryos exposed to copper compared to controls. There was no effect of copper on total glutathione. Expression of *nkx2.5* as one of the essential genes for the formation and development of the heart increased significantly; approximately 10 fold in the presence of Cu+Ca in comparison to the unexposed control or Cu exposure alone. Whereas expression of *mt2* increased significantly 6 fold compared to the control during Cu exposure without added Ca^{2+}. The second experiment (Chapter 4) aimed to investigate the effect of the dissolved Ag^+ as $AgNO_3$ on the survival of the early life stage of zebrafish. Embryos < 2 hpf were exposed to silver 0 (no added Ag), 2.5, 5, 7.5, 10 and 15 µg L^{-1} Ag as $AgNO_3$ for up to 72 h. Although, the survival was not affected by increasing concentrations of total silver, a decrease in hatching and increase in heart beat was observed. A significant increase in embryonic silver accumulation in both live embryos (at 24 and 72 hpf) and dead embryos (at 24 hpf) was observed. The accumulation of silver in 24 hpf live embryos was more significant than in dead embryos. Dead and live embryos at 72 hpf exposed to Ag had lower Na^+ and K^+ concentrations. Live embryos also showed a transient increase in Ca^{2+} concentration at 24 h. Four fold increases in Na^+K^+-ATPase activity, *Mt2*, and total glutathione concentration were seen in embryos after 72 h of exposure to $AgNO_3$ compared to controls. In contrast, *nkx2.5* gene expression was significantly decreased by 3 fold in 24 h aged embryos exposed to silver compared to controls. Due to the lack of studies that investigate the effect of silver on protein expression profiles during the early stages of development of zebrafish, the third experiment (Chapter 5) aimed to investigate the effect of silver on the changes of the expressed proteins of zebrafish embryos at the segmentation stage (24 hpf). The proteomics analysis successfully identified total of 810 proteins in the embryonic homogenate and quantified changes in their abundance in response to silver exposure. MS analysis showed the induction of new proteins which were absent

in control embryonic homogenates. Also the analysis revealed there were increased expression of proteins such as zona pellucida glycoprotein, ATP synthase subunit α and β, stressed proteins such as metal chaperones and heat shock proteins, antioxidant proteins such as catalase (CAT), superoxide dismutase (Cu-Zn), glutathione S-transferase M, and glutathione S-transferase and proteins related to muscular development such as myosin heavy polypeptide 2, actin alpha 1 skeletal muscle, slow myosin heavy chain 1, actin cytoplasmic 1, and tropomyosin proteins. Overall, the thesis confirmed that the early life stages of zebrafish are sensitive to metals and that there are critical windows of toxicity during development. Metal exposure at early stages of the development initiate several disturbances in biochemical processes as well as changes in molecular biology that affect fish survival.

Dedication

THIS WORK IS DEDICATED TO MY BELOVED Wife AHLAM AND MY CHILDREN,

SERAR, SALSAL, SERENE, SARI

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Abbreviations

ADP	Adenosine diphosphate
ADME	Absorption, distribution, metabolism, and excretion
ATP	Adenosine triphosphate
ATPase	Adenosine triphosphatease
Ag	Silver
Ag-Nps	Silver nanoparticles
$AgNO_3$	Silver nitrate
ANOVA	Analysis of variance
BLM	Biotic ligand model
bw	Body weight
Ca	Calcium
$CaCO_3$	Calcium carbonate
CAT	Catalase
CCs	Chloride cells
Cd	Cadmium
$CdCl_2$	Cadmium chloride
CdSe	Cadmium selenite
Cd Te QDS	Cadmium telluride quantum dots
CEB	Centre of environmental biotechnology
Cl^-	Chloride
Cu ATPase	Copper adenosine triphosphatease
CO_3	Carbonate
COX-17	Cytochrome *c* oxidase-17 copper chaperone
Cr	Chromium
Ctr 1	Copper transporter-1
Cu-Nps	Copper nanoparticles
$CuSO_4$	Copper sulphate

DCT 1	Divalent cation transporter 1
DNase	Deoxyribonuclease
DNA	Deoxyribonucleic acid
DO	Dissolved oxygen
DOC	Dissolved organic carbon
DOM	Dissolved organic matter
dpf	Days post fertilization
DTT	Dithiothreitol
dw	Dry weight
EDTA	Ethylenediamintetraacetic acid
EGPF	Enhanced green fluorescent protein
EtOH	Ethanol alcohol
GIT	Gastro intestinal tract
GPX	Glutathione peroxidase
GR	Glutathione reductase
GR-ir	Glucocorticoids receptor-immunoreaction
GSH	Glutathione (reduced)
GSSG	Glutathione (oxidized disulphate)
GST	Glutathione S transferase
H_2O_2	Hydrogen peroxide
HB	Heart beat
HBCD	Hexa bromocyclododecane
HEPES	Hydoxy methyl piperizine-1 ethane sulfonic acid
HFBA	Heptafluorobutyric acid (buffer B)
Hg	Mercury
hpf	hours post fertilization
HSPs	Heat shock proteins
ICP-MS	Inductively coupled plasma- mass spectrometry
IL-6	Inter leukin-6

IL-1β	Inter leukin-1β
K	potassium
LC50	Lethal concentration 50
MDA	Malondialdehyde
Mg	Magnesium
MeOH	Methanol
MPA	Medroxyprogestroneacetate
MRE	Metal response element
mRNA	Messenger ribonucleic acid
MS	Mass-spectrometry
Mt	Metallothionein
MTF-1	Metal-regulatory transcription factor
Na	Sodium
NCBI	National center for biotechnology information
Ni	Nickel
Nm	Nanometer
OH^-	Hydroxyl group
OH•	Hydroxyl radical
OS	Oxidative stress
P	Statistical probability
Pb	Lead
PBS	Phosphate-buffered saline
ppm	Part per million
qPCR	Quick polymerase cycle reaction
RBPs	RNA binding protein
RIPA	Radio-Immunoprecipitation Assay
RNS	Radical nitrogen species
ROS	Radical oxygen species
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis

SEM	Standard error mean
SOD	Superoxide dismutase
Soc1	Facilitated loading of cytochromes with Cu (Cu carrier to cytochromes inside the mitochondria)
−SH	Sulfhydryl group
SO_4	Sulphate
TBARS	Thiobarbituric acid reactive substances
TFA	Trifluoroacetic acid
U	Uranium
Zn	Zinc

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  SETAC Europe 23rd annual meeting, "Building a better future: Responsible innovation and environmental protection", 12 -16th May 2013, Glasgow, U.K. Abstract book, No. WE055, p211.

- Sahib M. Bakir, R. Handy and T. Henry (2013). Effect of Dissolved Copper Exposure on Cardiac Development and Function in Zebrafish Embryos (*Danio rerio*). Annual Meeting of Ecotoxicology Research and Innovation Centre Plymouth University and Society of Environmental Toxicology and Chemistry UK Branch, 9-10 Sep, 2013, Plymouth lecture Theatre, Portland Square Building, Plymouth University, U.K.

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- Sahib Mohammad Bakir, Theodore B. Henry, Richard D. Handy (2015). The effect of waterborne silver on survival, ionoregulatory function, metallothionein and nkx2.5 inductions in zebrafish (Danio rerio) embryos. Society of Environmental Toxicology and Chemistry, SETAC Europe 25th annual meeting. "Effect modeling of environmental systems – extrapolation and prediction of species response", 3-7 May 2015, Barcelona. Spain. Abstract book, No. TU020, p239

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Author’s Declaration

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Signed.

Date.....

Chapter 1

General introduction

1.1. Introduction

Pollution has long been considered as an important world problem (Calabrese et al., 1973; Sevcikova et al., 2013). Welburn (1988) stated that pollutants or contaminants are chemicals present in the wrong place at the wrong concentration. Thus, water pollution may be considered as the deterioration in the quality of water to the point that it becomes hazardous to aquatic ecosystems (Cooper, 1993). Although the aquatic environment naturally contains very low concentrations of metals, these concentrations have increased as a result of the increasing natural and domestic industrial anthropogenic activities (Wepener et al., 2004). Aquatic ecosystems are considered as good reservoirs of chemical pollutants, such as mining discharges, increased industrial discharge, domestic waste emission of atmospheric deposition, and agricultural applications such as fertilizers and pesticides (Ji, 2008; Liu et al., 2010). Trace metals like copper, zinc, iron, silver, cadmium, lead, etc., are associated with aquatic ecosystem pollution (Hodson, 1988); although some of these trace metals like copper, zinc, and iron are essential in low concentrations for metabolism and normal growth of fish (Canli and Atli, 2003; Wöhrle and Pomogailo, 2003). These metals can be toxic when present in higher concentrations (Sanchez et al., 2005; Öner et al., 2009). Moreover, metals, unlike other pollutants, are non-biodegradable (Wepener et al., 2004) and convert to more or less stable toxic compounds (Rainbow and Luoma, 2011). They are deposited and assimilated in water and may be accumulated in higher concentrations in aquatic organisms than the ambient level (Adams et al., 2011). Thus, metal pollution is a serious contaminant in the aquatic environment and is recognized by the ability of metals to initiate deleterious biological effects (Calabrese et al., 1973; Bellas et al., 2004). Metals cause acute toxicity and sometimes bioaccumulate in different biological systems (Rainbow, 2007), which leads to different types of deleterious effects such as immunosuppression (Zelikoff, 1993; Betoulle et al., 2002); induction of different types of stress proteins such as cytoprotective, heat shock protein 70, and metallothionein (Hogstrand et al., 1991); oxidative stress (OS) (Hoyle et al., 2007); histopathological changes (Al-Bairuty et al., 2013); disruption of endocrine systems (Drevnick and Sandheinrich, 2003; Handy, 2003); and

mortality (Chow and Cheng, 2003; Johnson et al., 2007). Extensive studies for many years have been focused on critical effects of both the essential and non-essential trace metals in fish and other aquatic organisms due to their ecophysiological and toxicological effects (Vijayavel et al., 2007; Wang and Rainbow, 2008; Hernandez et al., 2011; Rainbow and Luoma, 2011; Lahman et al., 2015). Recently, engineered nanoparticles that are used in domestic appliances are considered as a new cause of aquatic ecosystem pollution. Nanometals and metals that are used in the synthesis of the domestic appliances reach the aquatic ecosystem and result in a number of toxic effects due to their accumulation in the tissues of the target aquatic organisms (Bury and Handy, 2010; Shaw and Handy, 2011).

Fish are one of the important components of human nutrition. Their constant exposure and sensitivity to pollutants, or other changes in the water, is an attribute that lends these animals as excellent biological indicators of acute and chronic metal pollution in aquatic ecosystems (Benson et al., 2007; Sevcikova et al., 2011). The early life stages (ELS) of fish appear more sensitive to water pollutants than adults, with effects on their survival rate, hatching, growth (Scudder et al., 1988; Jezierska et al., 2009), and morphology of embryos (Dave and Xiu, 1991).

Zebrafish (*Danio rerio*) embryos have become a more attractive model for the detection of potential developmental toxicity of heavy metals and other pollutants. They are small-sized, develop outside the mother, are easy to maintain, and their transparency helps to follow the organogenesis and development of the organs. Moreover, being genetically similar to other vertebrates make the embryos preferable for studying the behaviour of gene transcriptions. Zebrafish show important aspects of vertebrate development and the evolution of the organs (Busch et al., 2011; Strähle et al., 2012; Yang et al., 2009).

1.2. Metals

Metals are categorised as heavy or trace metals, which are naturally occurring metallic elements recognized by high atomic weight and a density that is at least five times greater than that of water (Tchounwou et al., 2012). Trace metals are important contaminants of aquatic ecosystems. They are found naturally in the aquatic environment as a result of weathering and erosion (Viljoen, 1999), as well as from industrial processes (Biney et al., 1994). Elevated metal concentrations in aquatic ecosystems have been observed due to the rapid growth of the human population, increased industrialization, increased urbanization and socio-economic activities, exploration and exploitation of natural sources, extension of irrigation, modern agricultural practices, and lack of environmental regulation (Biney et al., 1994). Metals can be functionally classified according to their biological importance into essential and non-essential trace elements. The essential ones like copper and zinc play an important role as cofactors for numerous enzymes (Hogstrand et al., 1996b; Linder and Hazegh-Azam, 1996). However, higher concentrations of these metals are detrimental and initiate toxic effects in aquatic organisms such as the induction of OS due to generation of reactive oxygen species (Handy, 2003; Grosell et al., 2004; Qu et al., 2014) (Table 1.1). In contrast, the non-essential trace elements like cadmium have no biological function and moreover are also major pollutants of the ecosystems (Webb, 1979; Basha and Rani, 2003).

1.3. Toxicity of trace metals

Among the pollutants, metallic transition metals are the main cause of aquatic ecosystem contamination (Hodson, 1988), which initiate OS in aquatic organisms through the generation of ROS. This leads to the appearance of DNA strand breaks and causes apoptosis and cell death (Sanchez et al., 2005; Lushchak, 2011; Sevcikova et al., 2011). Biotic and abiotic aquatic ligands are considered to determine the speciation and bioavailability of metals and thus reduce their toxicity (Di Toro et al., 2001; Erickson et al., 2008).

1.3.1. Metal speciation and bioavailability

Fish and other aquatic organisms are good recipients of pollutants including metals. Heavy metals, unlike other pollutants in ecosystem, are non-biodegradable and recognized as being highly toxic even in small concentrations in the ambient. Due to their ability to accumulate in different vital organs of the organisms, they are enough to initiate several deleterious effects (Wright and Welbourn, 2002; Wepener et al., 2004). However, the relationship between the speciation and the bioavailability of the metals plays an important role in determination of metal toxicity (Markich et al., 2002). The ability of the metals to bind to the cell membrane and then transport through the cell membrane to the target tissues depends mainly on the speciation of the metal and the physicochemical factors of the environment (Markich et al., 2002). Metal toxicity can be influenced by the route of exposure (water, dietary, and injection) and the physical state of the metal (solid, liquid, and vapour). Speciation and bioavailability are also affected by biotic factors that are related to the organism in terms of species, stage of development, size of the organism, and the nutritional state, and abiotic factors that are related to the environment such as pH, temperature, alkalinity, salinity, and dissolved oxygen, and both affect the LC_{50} value as a result of the interaction between the abiotic factors and toxicants (Wang, 1987; Van der Merwe et al., 1993; Hansen et al., 2002; Hattink et al., 2006).

1.3.2. Water chemistry and metal toxicity

The importance of water chemistry has long been studied by many researchers. The free metal ions as bioavailable chemical species are affected by abiotic factors mentioned above (Cusimano et al., 1986; Bervoets and Blust, 2000; Pyle et al., 2002; Erickson et al., 2008).

Temperature is one of the abiotic factors that affect metal toxicity. It is well known that most aquatic organisms are poikilothermic (Hilmy et al., 1987). Van der Merwe et al. (1993) observed that rise in the temperature of the exposure

water increases the copper toxicity in adult and juvenile *Clarias gariepinus.* Increased temperature leads to increase in the rate of ion diffusion and the acceleration of the chemical reactions that result in incoordination and imbalance of various vital processes (Reynolds and Casterlin, 1980).

Water pH it is well recognized that firstly changes the speciation and bioavailability of the metal, and secondly, it changes the sensitivity of the cell surface, which affects the uptake and toxicity of the metals (Campbel and Stokes, 1985). A decrease in the pH affects the accumulation of metal within the organisms as a result of increased bioavailability of free metal ion concentrations (Markich et al., 2002). At low pH, the competition between increased H^+ concentrations as a proton with free metal ions for the same binding sites prevents the adsorption of the metal ions to the binding sites of the epithelial surfaces reduce metal toxicity (Pynnönen, 1995). On the other hand, pH alters the speciation of dissolved metals. Bury and Handy (2010) observed increased free Cu ions at low pH that became more toxic to teleost fish. It was observed that like copper, the acidic water is also toxic to fish (Wood and McDonald, 1982) (Shaw et al., 2012). Although both are similar in their toxicity, the combination between Cu and acidic water led to reduction in the toxicity of Cu due to the competition between increased H^+ and Cu^+ to bind the same sites in the gill epithelium (Laurén and McDonald, 1985). Increased mortality was observed at low pH during the exposure of fresh water fish to higher copper concentrations (McDonald, 1983). In addition, it was observed by Çoğun and Kargın (2004) there was higher copper accumulation in the liver at low pH.

The ameliorating effect of hardness is well known. Particularly the presence of Ca^{2+} as a main cation of hardness affects the toxicity and uptake of Cu, and other heavy metals and considered as the major physicochemical factor that affects the toxicity of heavy metals in fish (Miller and Mackay, 1980; Pagenkopf, 1983; Richards and Playle, 1999; Abdel-Tawwab et al., 2007; Chen et al., 2012). Addition of calcium before copper exposure mitigates the severity of Cu toxicity in gill, liver, GIT, and muscles of *Pelteobagrus fulvidraco* (Chen et al., 2012). The reduction in H^+ ion concentration and Ca^{2+} level in alkaline soft and ion-poor water leads to increase in the copper toxicity as a result of increased

copper binding to the gill surface membrane receptors (Meador, 1991), whereas in juvenile rainbow trout, Laurén and McDonald (1986) observed no effect of water hardness and alkalinity in the uptake of copper, while copper uptake reduced about 50% at pH 5.0.

Many studies have shown the Cl^- is a more protective agent than Ca^{2+} in reducing Ag toxicity (Hogstrand et al., 1996a; Bury et al., 1999). Low influence (1.5 fold increase) on the 96 h LC_{50} value was observed during increasing water [Ca^{2+}] from 50–2000 µM in the exposure of rainbow trout and in fathead minnows to 3.2–40 mg $AgNO_3$ L^{-1} (Bury et al., 1999), whereas Karen et al. (1999) observed in laboratory assays that the addition of $CaCO_3$ only offered a little protection against silver toxicity in rainbow trout, fathead minnows, and water fleas (*Daphnia magna*).

Humic acid is one component of DOM, reported as an important parameter influencing the metal bioavailability in the aquatic ecosystem through the reduction of the adsorption of the metals to the cell membrane (Livens, 1991; Erickson et al., 1996). The physico-chemical properties of humic acid are related to the presence of two oxyfunctional groups (carboxylic and phenolic hydroxylic groups) The oxyfunctional groups control the toxicity of the heavy metals by modifying their chemical speciation via the formation of complexes with metal cations, thereby influencing their transport and bioavailability and thus reducing the toxicity and bioavailability of the free metal ions to the biota by reducing their concentration in the water (Hart, 1982; Wang, 1987; Penttinen et al., 1995).

1.3.3. Bioaccumulation of heavy metals

Aquatic organisms tend to accumulate metals whether they are essential or not when present in a high concentration or non-essential present in low or high concentrations within their tissues (Chapman et al., 1996; Rainbow, 2007; Debelius et al., 2009). Bioaccumulation can be defined as the accumulation of the pollutants in the organisms when their absorption is greater than their

excretion (Bryan et al., 1979). Metals are easily dissolved and absorbed as free ions by fish and other aquatic organisms. Small concentrations of dissolved free ions can be toxic when their concentration within the aquatic organs is higher than the ambient (Paquin et al., 2000). The free ions to be accumulated within the specific organs must be available as a solute and get adsorbed in the cell surface as a first step and then across the cell membranes (Handy and Eddy, 2004). There are several factors that affect the adsorption, such as the concentration of free metal ions, number and type of solute (free ions), binding ligand on the epithelial surface, the rate of ion uptake and any associated replacement of surface ligands, and unstirred layer formation on the extracellular surface of the cell membrane (Handy et al., 2002). Other factors like the ability of fish to absorb the metals via the gills and across the skin and digestive tract and the role of the metals during the distribution in different organs of the organisms appear to play a role in the accumulation of metals (Erickson et al., 2008).

The uptake of the metals seems more important due to the route of entrance of the metals into the aquatic organism. There are different routes by which the fish can take up the metals, such as water passage through the gills that forms the normal passage of water during respiration, and considered as the main site of entrance, and then transport of the metals to the internal organ which occurs via the circulatory system (Evans, 1987). Food represents the second route of entrance of metals via mouth to the gastrointestinal tract (Bury et al., 2003).

The presence of cellular carriers that mediate the transport of metals across biological membranes are considered necessary for metal uptake mechanisms (Handy and Eddy, 2004; Bury and Handy, 2010). Absorption, distribution within the tissue, metabolism or storage, and then excretion (ADME) represent the internal factors that affect the accumulation of the metals in the target organs (Handy et al., 2008c). Gills, as the main osmoregulatory organ, reflect the ambient metal concentrations further in the liver, kidney, spleen, intestine, and muscles, which are considered as the probable organs for metal accumulation (Allen, 1994; Grosell et al., 2004; Shaw and Handy, 2006).

Differences in metal type, route of uptake, and the time of exposure are reflected in the accumulation of the metals within different organs. It has been observed that higher accumulation of cadmium in the kidney followed by gills and liver during the exposure of rainbow trout fish to cadmium with water, in comparison with dietary exposure, higher concentration of cadmium found in gut tissues followed by the kidney, liver and gills (Chowdhury et al., 2005). Handy et al. (1999) observed higher accumulation of copper in the intestine and liver with low concentration in the gills during the oral exposure of rainbow trout to copper. In adult fish, external epithelia of gill, skin, and gastrointestinal tract as well as the mucus on the epithelial surfaces as diffusion barriers all are considered as major barriers to the absorption of metals (Mallatt, 1985; Handy et al., 2005). The increase of the mucus secretion changes the environment at the epithelial surface and helps chelate and precipitate heavy metals (Playle and Wood, 1989; Karthikeyan et al., 2007).

Kidneys and gastrointestinal tract are considered as a part of the normal routes of the elimination of metals from the body (Deb and Fukushima, 1999). Further, bile in digestion and absorption of lipids from the intestine also contribute in the elimination of the metals, as in the case of accumulation of Cu in the liver and blood, which has no possibility to be excreted by the kidneys (Grosell et al., 1997; Grosell et al., 2000). Conjugation of Cu with increased plasma proteins as in case of increased plasma ceruloplasmin levels that are tightly bound to Cu reduces their accessibility to be excreted by the kidney (Grosell et al., 1998). On the other hand, the gills of the fish may be able to play a role in the excretion of metal ions from the systemic circulation by active efflux on the branchial ion transport pathway. It was suggested by Hickman Jr (1968) that gills, as an external route of excretion, are able to remove 60% of swallowed calcium.

Although the chorion is a protective structure surrounding the embryo, it acts as a route of metal transport. This protective structure is unable to protect the embryos against the penetration of heavy metals particularly during the swelling of the eggs due to absorption of water by perivitelline space (Peterson and Martin-Robichaud, 1982). During the early stage of embryonic development and immediately after fertilization, the embryo is surrounded by vitelline membrane,

enveloping layer and the chorion as major protecting structures against the pollutants (Finn, 2007). The perivitelline fluid (PVF) that occupies the space between embryo and chorion contains protein, lipid and carbohydrate, which form a colloidal suspension. The absorption of water by colloidal suspension of perivitelline space leads to the swelling of the egg, and as result of the swelling, the chorion becomes highly permeable to metal ions, which then enter the egg and cause a change in the structure and permeability of the chorion. Moreover, the PVF, due to its protein content, particularly glycoproteins, acts as an important trap for cations (Peterson and Martin-Robichaud, 1982; Blaxter, 1988; Finn, 2007). The presence of anionic glutamic sites on the chorion and significant amount of cysteine with high binding affinity of sulfhydryl group metals plays a probable role to bind selectively with the metal cations (Rombough, 1985; Sugiyama et al., 1996; Bell and Kramer, 1999; Guadagnolo et al., 2000). Both fish embryo and larvae are able to accumulate heavy metals. Many studies have confirmed that larval fish are more sensitive to heavy metals than embryos, depending on the toxicity of the heavy metals and developmental stages as well as the species of the organisms (Eaton et al., 1978; McKim et al., 1978; Dave, 1985; Scudder et al., 1988; Hutchinson et al., 1998; Kazlauskienë, 2002; Finn, 2007). The increase in the accumulation of metal in the whole embryo might be initiated firstly by the binding of metal ions to the sulfhydryl groups and then entering the intact embryo (Bell and Kramer, 1999). The existence of ion-transporting cells (ionocytes) on the yolk sac membrane, which are controlled by transmembrane proteins such as $Na^{+}K^{+}$-ATPase, may assist in the transportation of metals like copper into the embryo (Sucré et al., 2010). The increase in concentrations may be mediated by other mechanisms of copper entry as in adult tissues, such as Na^{+} dependent transporters, divalent metal transporter1 (Dmt1), and copper transporter -1 (Ctr1), which may provide possible mechanism of transport during the early stage of embryogenesis (Sharp, 2003; Mackenzie et al., 2004). The sensitivity of embryos to heavy metals is affected by several factors such as the embryonic development stages and time of exposure (McKim et al., 1978; Kazlauskienė and Stasiūnaitė, 1999; Jezierska et al., 2009), physico-chemical characteristics of water, concentrations of heavy metals, and species of fish (Wang, 1987).

1.3.4. Trace metals as inducers of oxidative stress

As heavy metals are major contaminants of the aquatic ecosystem, they promote OS (Stohs and Bagchi, 1995; Knight, 1998; De Wolf et al., 2001; Valavanidis et al., 2006; Nishida, 2011; Sevcikova et al., 2011). OS is defined as a state of increased production of oxidants more than the antioxidants' defence and repair system (Sies, 1985, 1997; Patel et al., 1999). Imbalance between the production of oxidants and the antioxidant defence system in living organisms is referred to as OS (Davies et al., 1994; Nishida, 2011). Increased lipid peroxidation, protein oxidation, DNA damage, cell membrane damage, and enzyme inactivity all are the probable effects of OS (Dalton et al., 1999; Stohs et al., 2001; Singh et al., 2006).

A free radical is an atom or molecule containing one or more unpaired electron and possesses significant degree of reactivity (Halliwell, 1994; Beneš et al., 1999). In the biological field, there are two types of free radicals, most of reactive oxygen species ROS and reactive nitrogen species (RNS) (Halliwell, 1994; Patel et al., 1999; Lushchak, 2014). Both are produced continuously in living cells, and they are essential to control normal physiological function in living organisms. At the same time they are responsible to potentiate deleterious effects when their levels exceed the level of cellular antioxidants (Valavanidis et al., 2006; Valko et al., 2006). ROS such as superoxide ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), hydroxyl radicals (HO•), and singlet oxygen (1O2) are the most important classes derived from oxygen during normal aerobic metabolism (Beneš et al., 1999; Inoue et al., 2003; Lushchak, 2011). There are two sources of ROS production; endogenous cellular sources include mitochondria as the main site of ROS production particularly during mitochondrial respiration, metabolism via cytochromes P450, peroxisomes, and inflammatory cell activation as in the case of phagocytosis activity of neutrophils and macrophages (Babior, 2000; Inoue et al., 2003; Valko et al., 2006), while exogenous sources include the potential substances that enable the initiation of the production of ROS, such as transition metals ions, pesticides, petroleum pollutants, and nano materials (Federici et al., 2007; Slaninova et al., 2009; Lushchak, 2011). According to the Haber-Weiss reaction, hydrogen peroxide

(H_2O_2) and superoxide ($O_2^{\bullet-}$) are the sources of hydroxyl radicals (OH•), as the reaction occurring in the cells normally requires the presence of catalyst to achieve the reaction, which is considered as a possible source of OS (Kehrer, 2000).

$O_2^{\bullet-} + H_2O_2 \longrightarrow HO^{\bullet} + O_2 + HO^-$

The presence of low concentrations of iron as transition metal ions in the cell plays a role as a catalytic factor to activate the reaction through the reduction of ferric to ferrous state (Fenton reaction) as a first step:

$Fe^{3+} + O_2^{\bullet-} \longrightarrow Fe^{2+} + O_2$

$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH^- + OH^{\bullet}$

The net reaction:

$O_2^{\bullet-} + H_2O_2 \longrightarrow HO^{\bullet} + HO^- + O_2$

The presence of other transition metal ions are capable to catalyst, such as in reaction via Fenton reaction (Kehrer, 2000).

$M^{n+} + H_2O_2 \longrightarrow M^{(n+1)} + OH^{\bullet} + OH^-$

where M^{n+} can be Ti^{3+}, Cu^{+}, Fe^{2+}, Co^{2+} and Cr^{4+} (De Wolf et al., 2001).

Hydroxyl radicals, as potent oxidizing radicals, possess the ability to react with biological molecules found at the site of formation initiating the oxidative damage.

Transition metals such as copper, chromium, cobalt, titanium and their complexes are capable of producing free radicals via reactions analogous to the Fenton reaction (Halliwell, 1994; Lushchak, 2011). In contrast, other transition metals without redox potential like mercury, nickel, lead, and cadmium cause OS in ways other than Fenton reaction via the reaction with –SH group. They impair the antioxidants particularly by interaction between metal ions and antioxidants sulfhydryl groups −SH (Gstraunthaler et al., 1983; Stohs and Bagchi, 1995). The increased free radical levels with decrease in free sulfhydryl group increases the possibility of OS formation (Gstraunthaler et al., 1983; De Wolf et al., 2001). On the other hand, it is well known that metal nanoparticles (NPs) are considered as the key for the generation of OS. The oxide nanoparticle forms of some transition metals such as titanium oxide (TiO2), zinc oxide (ZnO), cerium oxide (CeO2), copper oxide (CuO), and silver NPs

generate OS due to their deposition on the surface or inside the organelles of the cell (Buzea et al., 2007; Fahmy and Cormier, 2009; Shvedova et al., 2012; Manke et al., 2013).

Living organisms protect themselves against the generation of OS due to exposure to heavy metals and other toxic materials; they initiate a potential defence system of antioxidants. The antioxidants' potential defence system acts as a scavenger of ROS. Enzymatic antioxidants are of two types such as SOD, CAT, and GPX (MatÉs et al., 1999), and non-cellular enzymatic antioxidants include vitamin C, vitamin E, lipoic acid, carotenoids, flavonoids, selenium, and thiol antioxidants like GSH and thioredoxin system (McCall and Frei, 1999; Valko et al., 2006).

In fish, environmental pollution plays a crucial role in OS induction. Many studies have been focused on the formation of OS in fish during their exposure to heavy metals (Stohs and Bagchi, 1995; Kelly et al., 1998; Valko et al., 2005; Lushchak, 2011).

Table 1. 1 Toxic effect of metals on the aquatic organisms

Types of metals	Concentration/ Time	Types of Exposure	Species	Effects or Response	Authors
Ag $AgNO_3$	10 µg L^{-1} For 6 days	Water	Rainbow trout *Oncorhynchus mykiss*	No metallothionein induction. Decline plasma Na^+ and Cl^-. While Ca^{2+} and K^+ not affected. Blood glucose increased -5 fold. Progressive metabolic acidosis. Arterial P_{O2} increased and P_{CO2} decreased to compensate metabolic acidosis.	(Wood et al., 1996a)
Ag $AgNO_3$	2 and 10 µg L^{-1} For up to 75 h	Water	Rainbow trout *Oncorhynchus mykiss*	At 10 µg L^{-1} 50% immediate inhibition of Na^+ and Cl^- branchial influxes reach 100% at 8 h. Effluxes of Na^+ and Cl^{-2} were less affected. Net loss of Na^+ and Cl^- across the gills with significant decrease in plasma [Na^+] and [Cl^-] as result of changes in unidirectional fluxes. Lesser similar effects were seen at 2 µg L^{-1}.	(Morgan et al., 1997)
Ag $AgNO_3$	25 µg L^{-1} For 48 days	Water	Zebrafish *Danio rerio*	Stress effects causes increased swimming activity and elevated mucus secretion and presence of sloughed mucus strands in the tank.	(Bilberg et al., 2011)

Al $AlCl_3$	50 µg L^{-1} For 96 h at pH 5.8	Water	Zebrafish *Danio rerio*	Significant increased (36%) in acetylthiocholine hydrolysis in the brain. Significant decrease in locomotor activity, evaluated by the line crossings number (25%), distance traveled (14.1%), maximum speed (24%), and there was increase in absolute turn angle (12.7%).	(Senger et al., 2011)
Cd	5, 50, 500 µg L^{-1} For 4 and 9 weeks	Water	European flounder *Pleuronectes flesus*	Low Cd for 96 h causes reduction in haematocrit, haemoglobin and red blood cell count. Lymphocytes number increased significantly as an indication of immunological defense in fish.	(Johansson-Sjöbeck and Larsson, 1978)
Cd $CdCl_2$	0, 0.01, 0.05 and 0.1 µg g^{-1} For 36 h	Injection	Goldfish *Carassius auratus*	*MtmRNA* level increased in brain, kidney, liver, and intestine, and *GPX mRNA* decreased in liver, kidney as dose dependent.	(Choi et al., 2007)
Cd	0.06 mg L^{-1} For 60 days	Water	Silver barb *Puntius gonionotus*	Increased *MT mRNA* in both liver and kidney. Peak of kidney MT after 28 days of exposure dropped when fish were removed to Cd-free water, whereas liver MT levels remained higher after removal of fish to Cd-free water.	(Wangsongsak et al., 2007)

Cd	0.01, 0.05 and 0.1 ppm. For 3,7, 11 days	Water	Pacific oyster *Crassostrea gigas*	Antioxidant enzymes mRNA increased significantly in the gill being time and concentrations dependent. At 0.1 ppm Cd, CAT and GPX increased up to 3 days and decreased at 7 days. While, SOD increased at 7 days and decreased at 11 days. Exposure to 0.05 or 0.1 ppm for 7 days causes increased in aspartate aminotransferase, alanine aminotransferase and hydrogen peroxide levels.	(Jo et al., 2008)
Cd, Cu, Zn, Pb	5, 10, 20 µmol L^{-1} For 14 days	Water	Freshwater fish *Oreochromis niloticus*	Liver catalase (CAT) influenced by Cd and Pb, and inhibited by Zn. No significant changes in CAT with Cu exposure. Liver alkaline phosphatase (AP) stimulated at 5 µM and significant inhibition at 10 µM. Intestinal and serum AP stimulated by all metals. Na^+K^+-ATPase activity of gill and intestine was inhibited by all metal exposures except 20 µM Pb causes increase in gill enzymatic system. Inhibition of muscle Ca-ATPase activity by all metal exposures, except Cu exposures.	(Atli and Canli, 2007)
Cd $CdCl_2$	50 µg L^{-1} For 14 days as intermittent and continuous exposure	Water	Blue mussel *Mytilus edulis*	Continuous exposure showed significant increase (≥ 2 fold) in tissue Cd accumulation compared to intermittent, 2 fold significant increases in hemocyte infiltration of digestive system in comparison to intermittent exposure.	(Amachree et al., 2013)

Co	0 – 50 mg L^{-1} For 96 h	Water	Zebrafish *Danio rerio*	After 12 h of exposure fertilization rate was decreased 6% and embryo survival to hatching decreased 60 % at 25 mg L^{-1}. Sperm DNA strand breaks as Co dose was detected at 13 days. DNA damage of sperm retuned normal after 6 days incubation in clean water.	(Reinardy et al., 2013)
Cr Vl	43.7 mg L^{-1} For 96 h	Water	Nile tilapia *Oreochromis spp.*	After 24 and 96 h: decreased in the levels of total glycogen, lipid and protein in the liver, muscle and gill. After 24 and 96 h organs pathologies were seen.	(Abbas and Ali, 2007)
Cr Vl	85.7 mg L^{-1} For 96 h	Water	Gold fish *Carassius auratus*	Oxidative stress, genotoxicity and histopathology were seen in the liver and kidney.	(Velma and Tchounwou, 2010)
Cu $CuSO_4$	0, 25, 100, 200 µg L^{-1} For 21 days	Water	Three spined Stickleback *Gasterosteus aculeatus*	Rapid and transient increase of antioxidants enzymes (CAT, SOD, GPX, GSH, GST), and depletion of GSH.	(Sanchez et al., 2005)
Cu $CuSO_4$	5.5 mg L^{-1} For 96 h	Water	Flying barb *Esomus danricus*	Changes in gill morphology and inhibition in catalase and superoxide dismutase activity.	(Vutukuru et al., 2005)
Cu $CuSO_4$	8, 15 µg L^{-1} For 48 h	Water	Zebrafish *Danio rerio*	After 48 h there were increases in cytochrome-*c* oxidase subunit 17 (COX-17), and catalase associated with increase in Cu load and protein carbonyl concentrations in gill and liver.	(Craig et al., 2007)

Cu	0.503 or 1.25 mg L^{-1} with and without CaO_2	Water	Nile tilapia *Oreochromis niloticus* (L.)	Mortality increased significantly, reduced feed intake, food conversion rate (FCR) increased significantly, higher total lipid and tissue damage as Cu dose increased. Ca pre exposure reduced Cu toxicity.	(Abdel-Tawwab et al., 2007)
Cu $CuSO_4$	185.75 mg L^{-1} For 96 h	Water	Nile tilapia *Oreochromis niloticus*	Changes in the gill, liver and kidney morphology were induced by Cu exposure.	(Kosai et al., 2009)
Cu	0.15 and 0.3 mg L^{-1} for 15 days	Water	*Synechogobius hasta*	Fatty liver syndrome induced as Cu exposure. Increased lipid and reduced protein content in whole body and liver. Change enzyme activity (SOD, CAT, SDH, PK, LDH, LPL and HL). Increased hepatic lipid peroxidation level. Impaired histological structure of gill and liver.	(Liu et al., 2010)
Cu $CuSO_4$	0.75 mg L^{-1} For 24 h	Water	Fingerlings *Catla catla*	Behavioral changes such as erratic swimming, restlessness and surfacing were induced by Cu exposure.	(Patel and Bahadur, 2010)
Cu	10 and 30 µg L^{-1} For 30 days	Water	Rainbow trout *Oncorhynchus mykiss*	Decreased of condition factor weight (CFW), specific growth rate (SGR), body weight gain (BWG). Increased food conversion rate (FCR) at the end of the experiment. At day 15 glucose, AST and ALT elevated, while triglyceride and cholesterol decreased.TP and ALP increased lineally by time and [Cu]	(Heydarnejad et al., 2013)

Cd Cu Zn	3 µg L^{-1} 75 µg L^{-1} 250 µg L^{-1} for 100 days (Chronic exposure)	Water	Rainbow trout	All have no effect on growth. Appetite increased in Cu exposure and decreased in Cd exposure. Swimming speed significantly lowered in Cu exposure associated with O_2 consumption at higher swimming speed. Branchial Na^+K^+-ATPase elevated with Cu exposure only not in Cd.	(McGeer et al., 2000b)
Cd Cu Zn Pb	5, 10 and 20 µmol L^{-1}For 14 days	Water	Fresh water fish *Oreochromis niloticus*	Liver catalase (CAT) influenced by Cd and Pb, while not affected by Zn and Cu. Liver alkaline phosphatase (AP) stimulated at 5 µM and significantly inhibited at 10 µM. Intestinal and serum AP stimulated by all Zn concentration and 10 µM Cu. Ca-ATPase inhibited by all except Cu exposure.	(Atli and Canli, 2007)
Pb	0, 100, 400 and 800 µg g^{-1} For 60 days	Food	Nile tilapia *Oreochromis niloticus*	ALT, AST and LDH activity decreased in kidney and stimulated in the liver. Inhibition of Alkaline phosphatase, Na^+K^+-ATPase, Ca and Mg-ATPase activities in liver and kidney as Pb concentration dependent. Fe, Cu and Zn of liver and kidney content decreased with increased Pb concentrations.	(Dai et al., 2009)

1.4. Copper

Rock and mineral mining are the natural sources of copper (Bowen, 1985). This metal is widely used in various industrial and domestic applications such as in electrical industries, air liquid filtration, batteries and thermal conductivity (Sloman, 2006), in wood preservatives as a chromated copper arsenate (Hingston et al., 2001), and as copper-borax (West, 2001) and as a pesticide in agricultural practices (Liu et al., 2010). In aquaculture, copper sulphate is used to control algae and some pathogens in water (Chen et al., 2006; Liu et al., 2010). Copper nanoparticles (CuNPs) are particles that have at least one dimension less than 100 nm (Roco, 2003), used in skin applications and textiles due to their bactericidal effects, and considered as xenobiotic sources of copper for aquatic organisms (Griffitt et al., 2007; Yoon et al., 2007; Heinlaan et al., 2008; Aruoja et al., 2009). Copper is one of the essential trace elements found in living tissues and exists in an oxidized state, cupric (Cu^{2+}), which is found abundantly in biological systems, or in a reduced state, cuprous (Cu^{+}) (Linder and Hazegh-Azam, 1996; Uauy et al., 1998). Copper has long been recognized as a cofactor in numerous metalloenzymes, particularly for enzymes that catalyse oxidation-reduction reactions such as cytochrome-*c* oxidase (mitochondrial respiration), superoxide dismutase (SOD) (antioxidant defence), ceruloplasmin (iron homeostasis), tyrosinase (melanin pigment synthesis), lysyl oxidase (development of connective tissue), dopamine monooxygenase (catecholamine production), and peptidylglycine α-amidating mono-oxygenase (neuropeptide hormones synthesis) (Linder and Goode, 1991; Pelgrom et al., 1995; Linder and Hazegh-Azam, 1996; Uauy et al., 1998; Peña et al., 1999; Linder, 2001). Although in low concentration, copper is an essential micronutrient for fish, but when present in concentrations more than the necessary requirement it leads to several biochemical and physiological changes such as survival disturbances, loss of appetite, growth retardation, ionoregulatory impairment, pathohistological alterations, and changes in serum chemistry (Handy, 1992; Marr et al., 1996; McGeer et al., 2000b; Burke and Handy, 2005; Shaw and Handy, 2006; Al-Bairuty et al., 2013; Heydarnejad et al., 2013).

Copper has received considerable attention particularly in humans. Imbalance of copper metabolism leads to two genetically fatal disorders related to the P-type ATPase transporter of Cu through the epithelial membrane known as Menke's and Wilson disease (Camakaris et al., 1999). In Menke's disease, the defect of the ATP7A gene as an isoform of Cu-ATPase disturbs the absorption of dietary Cu across the intestinal epithelium or disturbs the regulation of Cu in somatic epithelial cells (e.g. vascular endothelium, fibroblasts). In contrast, Wilson's disease is recognized as a defect in the ATP7β gene causing failure in Cu excretion from the body, which leads to severe copper toxicity (DiDonato and Sarkar, 1997; Camakaris et al., 1999; Menkes, 1999a, b)

1.4.1. Copper uptake and normal transport

Copper ions have a high affinity to interact with thiol groups on proteins (Letelier et al., 2005). Histidine, cysteine, and methionine-containing proteins have high affinity to bind with copper that leads to their inactivation (Camakaris et al., 1999). In fish, first, copper uptake from gill and intestine is initiated by electrostatic adsorption to the mucosal membrane; second, then is transferred to the gut cell crossing the plasma membrane via facilitated diffusion through ion channels depending on energy-dependent ATPase system; third, it is transferred inside the gut cell via binding to the selective proteins recognized by their ability to bind metal ions and known as metal chaperones; fourth, copper is exported from the cell to the blood against the electrochemical gradient (Handy et al., 2000; Handy et al., 2002; Burke and Handy, 2005; Vijayavel et al., 2007). External copper is mostly present in the cupric state (Cu^{2+}); before entering the gut cell, it is reduced to the cuprous state (Cu^{+}) by an endogenous plasma membrane reductase (McKie et al., 2001; Knöpfel and Solioz, 2002). Inside the cell (Figure.1.1), copper ions are bound to metallochaperone polypeptides, which are responsible for carrying Cu^{+} to many sites inside the cell that require Cu^{+}, such as Cu-dependent enzymes and biochemical processes. Polypeptide metallochaperones carry Cu^{+} to the Golgi vesicles against the concentration gradient via the Cu^{+}-ATPase (Bury et al., 2003). Via exocytosis, Golgi vesicles carry copper ion to the serosal membrane for release (Bury and Handy, 2010).

Copper in the cuprous state (Cu^{+}) is effluxed to the blood from the intestinal cells via the Cu/Cl^{-} symporter. The route is dependent partly on the luminal and serosal Cl^{-} concentration (Handy et al., 2000). Other metallochaperones that are considered in the transfer of copper ion inside the cell include several carriers like homologous Atox1 that carry Cu^{+} to the ATPase in the Golgi, CCs that carry Cu^{+} to cytosolic SOD (Culotta et al., 1997; Lu et al., 1999; Lutsenko et al., 2002), Cox17 (an oligomer with three Cu binding domains) that transfer Cu^{+} to the inner mitochondrial membrane, while, Sco1, Cox1, and Cox2 carriers that facilitate loading of cytochromes with copper (Paret et al., 1999; Heaton et al., 2000; Heaton et al., 2001).

Metallothionein as a metal-binding protein and glutathione (GSH) are considered as copper chaperones, which are involved in intracellular transfer, storage and delivery of copper (Coyle et al., 2002). Metallothionein is induced by copper as well as other metals and is recognized as a necessary cellular component for the storage of copper as an essential micronutrient and sequestration of undesirable copper levels (Carpenè et al., 2007). The interaction between GSH and copper ion results in a stable complex Cu-GSH, which acts as a resident and major storage protein of copper ions before incorporation with metallothionein. Moreover, Cu-GSH complex efficiently acts as a donor of Cu ion to Cu/Zn SOD (Ferreira et al., 1993). The transfer of Cu in the blood depends mainly on the plasma protein ceruloplasmin in the form of $CuHis_2$ (Hilton et al., 1995; Luza and Speisky, 1996; Harris, 2000) or binding to albumin and other low molecular weight plasma proteins circulating in the blood (Harris, 2000).

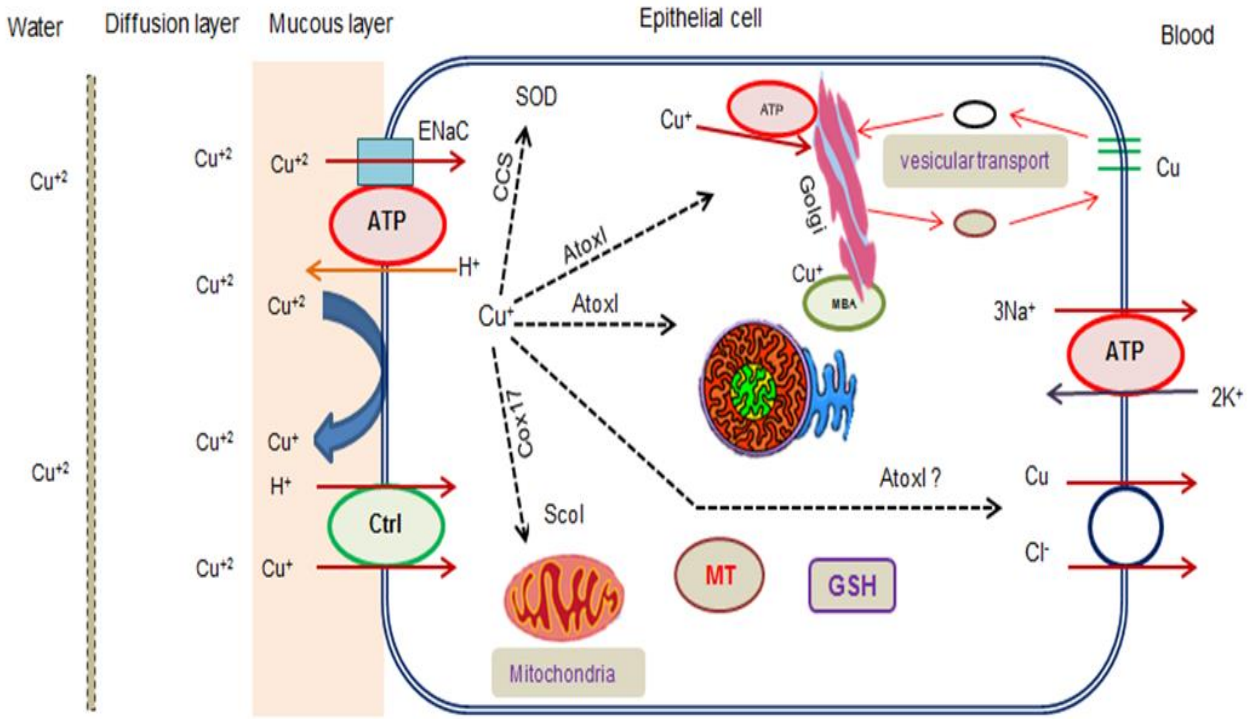

Figure 1.1 Schematic illustrations of copper uptake and cellular transport pathways across the plasma membrane of zebrafish gill and intestine apical cells. Briefly, to enhance the importance of the copper transfer to the cell via either copper transporter 1 (Ctr1) or putative epithelium Na channels (ENaC), within the cell membrane cupric ions (Cu^{2+}) are reduced to cuprous ions (Cu^{+}). Within cell cytoplasm, copper bind to several types of metallochaperones such as Atox 1 to drive copper to the Golgi apparatus via Cu-ATPase, and within the Golgi apparatus copper bind to metal binding protein (MBP). Copper releases to the blood via exocytosis through the movement of Golgi vesicles to the basolateral membrane of the cells. Atox 1 delivered Cu to the nucleus, CCs is the copper chaperone for the cytoplasmic superoxide dismutase (SOD1), Cox17 (delivered Cu to the inner mitochondrial membrane), Sco1 facilitated loading of cytochromes with Cu. Excess Cu could bind to low molecular mass protein such as glutathione (GSH) and metallothionein (MT).

1.4.2. Copper toxicity

Elevated environmental concentrations have shown a variety of negative effects on fish physiology such as ionoregulatory disturbance (Sola et al., 1995; Li et al., 1996; McGeer et al., 2000b; Grosell and Wood, 2002; Handy et al., 2002). Higher concentrations of copper initiate OS as a result of the increased generation of ROS. Impairing antioxidant enzymatic defense, particularly those involving thiol-containing antioxidant, such as GSH and enzymes like Na^+K^+-ATPase, protein oxidation, lipid peroxidation, alteration of gene expression, and changes in the redox state of the cells are the main adverse effects of increased Cu accumulation (Geret et al., 2002; Valavanidis et al., 2006; Company et al., 2008; Sevcikova et al., 2011). Inhibition of liver GSH as well as transient increases of antioxidant enzymes such as catalase (CAT), SOD, and GPX concomitant with Cu accumulation in the liver were observed by Sanchez et al. (2005) during the first eight days of the exposure of three-spined sticklebacks (*Gasterosteus aculeatus*) to high concentrations of Cu at 100 and 200 µg L^{-1} for three weeks. An imbalance of aerobic metabolism, decrease in mitochondrial SOD and CAT, and selenium-dependent and total glutathione peroxidase (GPX) concomitant with increased lipid peroxidation was seen in the first week in clam (*Ruditapes decussatus*) exposed to Cu (Geret et al., 2002). Inhibition of (CAT) and GPX activity may be explained as a result of the formation of $OH^{\bullet}$ from the reaction between Cu^+ and H_2O_2, which is regarded as a substrate of CAT and GPX (Geret et al., 2002).

On the other hand, increased lipid peroxidation as a marker of the oxidative damage was highlighted by many researchers. The dietary copper toxicity was studied by Baker et al. (1998) and Hoyle et al. (2007). The latter showed that the progressive rise of thiobarbituric acid reactive substances (TBARS) in the gill and intestine of African walking catfish (*Clarias gariepinus*) feed excess dietary Cu (1500 mg Cu kg^{-1} dw feed) for 30 days, was concomitant with total GSH pool as protective response against the oxidative damage was seen in the intestine and gill. Oxidative damage may vary in concentrations in tissues targeted by heavy metals. Significant differences in the concentration of TBARS

within the target tissues was observed by Shaw et al. (2012) in rainbow trout (*Oncorhynchus mykiss*) exposed to different concentrations of Cu as $CuSO_4$ and Cu-NPs. The difference varied with depletion of TBARS at concentration 20 µg L^{-1} in the gills and no changes were observed in the branchial during Cu-NPs exposure. The variation in the levels of some antioxidant enzymatic defences can be regarded as an indicator or marker of the oxidative damage. Increased malondialdehyde (MDA) level related to the increased lipid peroxidation was observed in the viscera of teleost fish (*Esomus danricus*) exposed to a concentration of 5.5 mg L^{-1} Cu, concomitant with appreciable decline in SOD and CAT activity (Vutukuru et al., 2006). Sáez et al. (2013) observed that the inactivation of most antioxidant enzymatic defence such as CAT, GST, SOD, GPX, GR, and DT-diaphorase (DTD) as a detoxifying enzyme coincided with decreased MDA value in *Gambusia holbrooki* exposed to sub-lethal Cu concentrations (0.10, 0.17, and 0.25 mg L^{-1}).

1.4.3. Role of copper in oxidative stress generation

Copper, as a transition heavy metal, plays a role in the formation of free radicals such as ROS (Stohs and Bagchi, 1995; De Wolf et al., 2001). Fish are capable of taking up copper from the water, and the excess accumulation in the target tissues occurs as a reflex of the elevated ambient copper concentrations. It is well known that excessive accumulation of copper leads to generation of OS (Laurén and McDonald, 1985, 1987; De Boeck et al., 2001b; Kamunde et al., 2002; Grosell et al., 2003; Grosell et al., 2004). Copper is a redox active metal that promotes the generation of ROS through redox cycling. The ionic copper and even the cupric or cuprous forms of copper make it more toxic and capable to produce ROS via the Fenton reaction (Halliwell, 1994; Peña et al., 1999; Powell, 2000; Craig et al., 2007; Lushchak, 2011). The reduction of the cupric state (Cu^{2+}) to Cu^{+} in the presence of superoxide ($O_2^{\bullet -}$) and reducing agents such as ascorbic acid or GSH leads the formation of hydroxyl radicals (OH•) from hydrogen peroxide via Fenton reaction.

$$O_2^{\bullet -} + Cu^{2+} \longrightarrow O_2 + Cu^{+}$$

$$Cu^{+} + H_2O_2 \longrightarrow Cu^{2+} + OH^{-} + OH^{\bullet}$$

1.5. Silver

Silver is one of the naturally occurring components of the surface water. Although natural leaching is considered as the main source of silver, its concentration is elevated due to anthropogenic activities such as silver mining and photo processing (Purcell and Peters, 1998; Wood et al., 1999), while agricultural and industrial applications are other sources of silver pollutants (Morgan et al., 1997a). Recently, silver nanoparticles (Ag-NPs) have been widely used due to their antibacterial and antifungal agents in consumer products such as in water machine, biocide medicine, and water purification. They are considered as another source of contamination of aquatic environment due to the release of free silver ions (Ag^+), which further act as a source of contamination of the aquatic environment (Chen and Schluesener, 2008; Damm et al., 2008; Powers et al., 2010).

1.5.1. Silver toxicity

Silver nitrate ($AgNO_3$) is widely used in acute and chronic fresh water laboratory exposures and recognized by its high solubility and dissociation to release high levels of free ionic silver (Ag^+) (Guadagnolo et al., 2001). The ionic form of silver (Ag^+) is extremely toxic to aquatic organisms due to their bioavailability (Hogstrand and Wood, 1998; Gorsuch et al., 2003; Morgan and Wood, 2004; Wood et al., 2010). It is well known that the acute toxicity of Ag in aquatic animals in seawater is much lower than that in freshwater (Hogstrand and Wood, 1998; Shaw et al., 1998) due to the presence of high level of Cl^- concentration and other anion ligands like sulphide and DOM (Hogstrand and Wood, 1998), which bind to Ag^+ to eliminate their availability and toxicity (Wood et al., 1999). The presence of Cl^- in seawater as an abiotic factor reduces the bioavailability of free silver ions via the formation of an insoluble $AgCl_2$ complex and hence reduces the available amount of free Ag^+ (Ferguson and Hogstrand, 1998). The toxic effect of silver is exerted during the initial hours of exposure, which is considered as the critical period before silver ion forming complex with biotic ligand model (BLM) or the competition with various cations such as Na^+,

Mg^{2+}, Ca^{2+}, K^{+}, and Sr^{2+} on the binding sites on the gill, which act to modify the toxicity of silver (Janes and Playle, 1995; Schwartz and Playle, 2001; Bianchini et al., 2005). Although the sites of toxic metal binding on the gill and gut play a role in determination of silver toxicity, the presence of abiotic ligand model such as organic colloids, thiosulfate, sulphide, and particularly chloride (Cl^{-}) and dissolved organic carbon (DOC) change the speciation of silver through formation of silver complexes that act toward reducing the bioavailability of ionic silver (Janes and Playle, 1995; Morgan and Wood, 2004). Acute silver toxicity decreases with increase in alkalinity and pH, and the presence of carbonate in the water may decrease the bioavailability of free silver ions and hence decrease its toxicity (Erickson et al., 1998).

Silver, as a toxic metal, inhibits the activity of $Na^{+}K^{+}$-ATPase located at the basolateral membrane of gill epithelium, which appears sensitive to silver. The interaction of silver ions with specific sites on the $Na^{+}K^{+}$-ATPase molecules leads to disturbance of the osmoregulatory function and electrolyte levels in the whole body and particularly in the cardiovascular system, which leads to death of the aquatic organisms due to circulatory collapse (Hogstrand et al. 1996, Webb & Wood 1998, Grosell et al. 2000, Pedroso et al. 2007, Vijayavel et al. 2007, Wood et al. 2010). Morgan et al. (1997) documented the toxicity of silver to the carbonic anhydrase, which is present in the apical region of the gill epithelial cells, as well as to $Na^{+}K^{+}$-ATPase, which resulted in the inhibition of Na^{+} and Cl^{-} transport across the epithelial region of the gill cells.

1.5.2. Role of silver in oxidative stress generation

Silver induces the production of intracellular ROS, the way superoxide anion radicals lead to the generation of OS (Cortese-Krott et al., 2009). Like other transition metals, silver may assists in the generation of OS through the increase in the formation of ROS via the Fenton reaction to catalyse the production of oxyradical from oxygen, such as superoxide anion radicals, hydrogen peroxide, and hydroxyl radicals. On the other hand, within the mitochondria and via the reaction with −SH groups of the respiratory chain

enzymes, silver leads to production of superoxide anion radicals (Almofti et al., 2003). The ionic form of silver has high affinity to react with −SH groups forming stable complexes of hemi-silver sulphides and plays a role in impeding silver as well as other transition metal ions to reach the target organs, which causes cellular damage (Gurd and Wilcox, 1956; Passow et al., 1961).

1.6. Toxicity of trace metals to early life stages of fish

1.6.1. Effect of trace metals on the development and growth on ELS

The ELS of aquatic organisms are the critical and sensitive stages for water pollutants (Calabrese et al., 1973; Weis and Weis, 1991; Jezierska et al., 2009). Many studies have been focused on the toxicity of metals during ELS of fish (Table 1.2). The early stages of embryonic development appear sensitive to trace metals particularly after fertilization and before the egg chorion hardening (Jezierska and Slominska, 1997; Finn, 2007; Johnson et al., 2007; Jezierska et al., 2009; Kong et al., 2013). The egg chorion entirely protects the embryo against the penetration of heavy metals, particularly during the swelling stage of the eggs due to the absorption of water by perivitelline space (Peterson and Martin-Robichaud, 1982). In barbell (*Barbus barbus L*.) eggs, the swelling reaches up to a maximum of 10% during the first few hours after fertilization (Lugowska, 2009). It is well known that the disturbance of survival during the early stages of the development is the main effect of heavy metal intoxication. Higher embryonic mortality was observed within 24 hours post fertilization (hpf) particularly in blastula and gastrula stages (Weis and Weis, 1991; Chow and Cheng, 2003; Johnson et al., 2007).

The occurrence of high mortality at the early stage of embryonic development is related to the precise stage of the embryonic development and to the exposed metal species. It was seen that the newly fertilized eggs of the rainbow trout (*Oncorhynchus mykiss*) are more sensitive to nickel exposure than in the eyed stage (Nebeker et al., 1985). According to Samson and Shenker (2000), 15–20% mortality of zebrafish (*Danio rerio*) embryos exposed to methyl mercury had occurred at 8 hpf. Higher mortality was observed at the same time in zebrafish embryos exposed to Cd (Hallare et al., 2005). Fish larvae and early juveniles are more sensitive to copper in low concentrations, while embryo survival is affected by high concentrations (McKim et al., 1978; Scudder et al., 1988). The exposure of fish and other aquatic organisms at ELS to abnormal concentrations of copper has been suggested as being more toxic (Grosell et al., 2003; De Boeck et al., 2004), which causes reduction in survival, reduction in success and delay in hatching, and

causes retardation in growth and morphological deformity (Dave and Xiu, 1991; Ługowska and Witeska, 2004; Johnson et al., 2007; Cao et al., 2010a). Johnson et al. (2007) observed higher mortality of zebrafish (*Danio rerio*) embryos particularly before the hardening of the chorion was concentration dependent. The mortality in (53 µg L^{-1}) during first 5 hpf was increased to 100% in concentration (1099 µg L^{-1}). According to Cao et al. (2010b), higher mortality was seen in red sea bream (*Pagrus major*) embryos exposed to 0.08 mg L^{-1} Cu, and the result showed that the embryos are more sensitive than larvae to Cu toxicity. Exposure of embryos to silver ($AgNO_3$) at an early stage of development showed higher mortality. Guadagnolo et al. (2001) observed that the mortality reached 56% by day 32 post fertilization during chronic exposure of rainbow trout (*Oncorhynchus mykiss*) embryos to 13.5 µg L^{-1} silver in comparison to control and 0.1 and 1.2 µg L^{-1} concentrations mortality reached 33%, whereas the mortality of rainbow trout embryos in acute exposure reached 100% at recovery four days after exposure to silver concentration of 14.15 µg L^{-1} for 5 days between 8–17 days post fertilization (Guadagnolo et al. 2000).

1.6.2. Effects of trace metals on hatching success

Delayed and decreased successes in hatching have been observed in embryos exposed to heavy metals by many researchers. The process of hatching is a combination of biochemical (enzymatic) and mechanical mechanisms (De Gaspar et al., 1999; Fraysse et al., 2006). During the development of the embryo, hatching glands are developed. The glands are responsible for the synthesis and secretion of choriolytic enzymes known as chorionase, which are considered particularly as proteolytic factors that disintegrate the zona interna of the chorion (Yamamoto and Yamagami, 1975; Kapur and Yadav, 1982; De Gaspar et al., 1999). As chorionases are metalloproteases, trace metals disturb their normal function (Bourrachot et al., 2008). The enzyme reactions are followed by embryonic behavioural process (mechanical mechanisms) such as twisting movement of embryo to destroy the zona externa of the chorion and assist to release the embryo out of the chorion (Schoots et al., 1982; von Westernhagen, 1988; Fraysse et al., 2006).

Decreased hatching was observed as being dependent on copper concentration during exposure of zebrafish embryos to concentration of 50 up to 1000 µg L^{-1} Cu for 72 hpf (Johnson et al., 2007), whereas 36–61% of red sea bream embryos hatched between 48–72 hpf when exposed to copper concentration ≥ 0.08 mg L^{-1} (Cao et al., 2010a). Low hatchability 0–90 % was seen in red sea bream exposed to cadmium concentration ≥ 0.8 that µg L^{-1}reached to 97–100 % in 2.4 mg L^{-1} in 48 h (Cao et al., 2009). Hatching rate of zebrafish embryos decreased significantly as it is dose dependent in the presence of palladium (Pd) in the concentration range of 50 up to 450 µg L^{-1} (Chen et al., 2015). Delayed hatching due to the lowering in chorionase level was observed in *Cyprinus carpio* embryos by Mis and Bigaj (1997) during the exposure of embryos to zinc or copper, while Dave and Xiu (1991) reported that the weakness of muscular movement is the cause of delayed hatching in zebrafish (*Danio rerio*) embryos exposed to mercury, copper, nickel, lead, and cobalt. Delayed hatching was observed in embryos that were exposed even to low concentrations of copper. According to Dave and Xiu (1991), delayed hatching occurred during the exposure of zebrafish embryos to a low concentration of 0.002 µmol L^{-1} Cu. Palmer et al. (1998) observed that the lowest copper concentration of 0.17 µmol L^{-1} causes delayed hatching, whereas Johnson et al. (2007) recorded decreased hatching success during the exposure of zebrafish embryos to low concentrations of 50 µg L^{-1} Cu and above. In contrast, delayed hatching by 2–3 days was seen during the chronic exposure of rainbow trout (*Oncorhynchus mykiss*) embryos to concentrations of 0.1 and 1.0 µg L^{-1} Ag in the presence of DOC, respectively (Brauner and Wood, 2002b). Delayed hatching and reduced embryonic survival were recorded as dependent on silver concentrations by Powers et al. (2010) in zebrafish exposed from 0–5 days to Ag^{+} as $AgNO_3$ concentrations 0.01, 0.1, 0.3, 1.0, 3.0, 10.0, and 100 µM for 5 days. At 1 µM, embryos showed delayed hatching only, whereas reduced embryonic survival and delay hatching were observed with ≥ 3 µM.

1.6.3. Effect of heavy metals on cardiac function

Heart is an excitable tissue that depends on electrolyte fluxes for physiological function. It is also intolerant of OS/hypoxia. The fish cardiovascular system at ELS,

like other organs, is a target of heavy metal exposure. Cardiac cells are excitable cells like skeletal and smooth muscle cells, which require constant levels of electrolytes to maintain their normal function (Hille, 2001). Na^+K^+-ATPase is an essential enzyme to maintain the intracellular physiological process of the cells in adult animals through maintaining the normal intra and extracellular electrochemical gradient (Shou et al., 2003). The interaction with Na^+/Ca^{2+} is important to regulate the cardiac function through maintaining the normal cardiac electrochemical gradient (Schwinger et al., 2003).

In zebrafish, there are three isoforms of Na^+K^+-ATPase, α1, β1, and α2, all of which are important for the development of heart according to their function in heart development (Shu et al., 2003). The importance of Na^+K^+-ATPase α2 isoform for establishing cardiac laterality is well known, whereas α1, β1 isoform is important for the extension of the primitive heart tube and differentiation of the cardiomyocyte (Shu et al., 2003). The presence of sulphydryl −SH group as a constituent of the enzyme is targeted by free metal ions, which show a high affinity to bind covalently with −SH groups that leads to inhibition of the enzyme activity (Kone et al., 1990; Li et al., 1996).

The disturbance of the cardiovascular system by exposure to heavy metals has been studied by many researchers (Cheng et al., 2001; González-Doncel et al., 2003; Cao et al., 2009; Li et al., 2009; Barjhoux et al., 2012). The heart is the first organ of the cardiovascular system to form and function in fish embryos (Glickman and Yelon, 2002; Targoff et al., 2008) and maybe effected by metals. For instance, significant increase in heart rate was seen at 28 hpf in exposed zebrafish embryos with increase in copper concentrations (Johnson et al., 2007), whereas decrease in heart rate was observed in common red carp (*Cyprinus carpio*) embryos exposed to a concentration of 0.05 mg L^{-1} Cu (Stouthart et al., 1996). According to Cao et al. (2010b), the heart rate of red sea bream (*Pagrus major*) embryos was not significantly affected by copper concentration, while the newly hatched larvae showed significant decreases in heart rate when exposed to a concentration of 0.08 mg L^{-1}. Although Japanese medaka (*Oryzias latipes*) embryos showed increased heart rate (tachycardia) during exposure to cadmium (Barjhoux et al., 2012), Cao et al. (2009) reported that the heart rate of red bream embryos was not significantly

affected when exposed to a concentration of 3.2 mg L^{-1} Cd, whereas the newly hatched larvae showed decrease in heart rate when exposed to a concentration ≥ 0.8 mg L^{-1} of Cd. In contrast, similarly, zinc concentration of 2.5 mg L^{-1} did not significantly affect the heart rate of red sea bream embryos, while increased heart rate in larvae was seen at concentration ≥ 1.0 mg L^{-1} (Huang et al., 2010).

Table1. 2. Summary of studies carried out into the toxicity of heavy metals to early life stages of fish

Metals	Concentration/Time	Species/embryo	Toxic effects	Authors
Ag ($AgNO_3$)	0.01, 0.3, 1, 3, 10 and 100 µmol (from fertilization-5 dpf)	Zebrafish *Danio rerio*	Significant reduction in survival at ≥ 3 µM. Delayed hatching at 1 µM. Delayed inflation of the swim bladder at 0.1 µM.	(Powers et al., 2010)
Ag ($AgNO_3$)	0, 0.1 and 10 µg L^{-1} + 12 mg $L^{-1}$of dissolved organic Carbone (DOC) +120 mg L^{-1} $CaCO_3$ / (from fertilization- 1 week post hatch)	Rainbow trout *Oncorhynchus mykiss* embryos and larvae	At 0.1 µg L^{-1} without DOC, significant growth reduction and increase in % daily mortality associated with ionoregulation disturbance, and reduction in whole Na. At 10 µg L^{-1} with DOC, reduced dissolved silver, significant decrease in % daily mortality up to hatch. DOC did not mitigate the ionoregulatory disturbance. Protection effect of DOC during chronic Ag exposure is less than in acute exposure.	(Brauner and Wood, 2002b)
Ag ($AgNO_3$)	0, 0.1 and 1.0 µg L^{-1} + 120 mg L^{-1} $CaCO_3$ (from – 1week post-hatch	Rainbow trout *Oncorhynchus mykiss* embryos and larvae	At 1.0 µg L^{-1} small significant increase in mortality (16%) relative to control, increased rate of growth and ionoregulatory development. At 0.1and 1.0 µg L^{-1} unidirectional Na^+ uptake increased just prior to and following hatch. Change in Na^+K^+-ATPase activity was observed.	(Brauner and Wood, 2002a)
Ag ($AgNO_3$)	0, 0.1, 1 and 10 µg L^{-1} (chronic exposure)	Rainbow trout *Oncorhynchus mykiss*	At 10 µg L^{-1} high mortality (56%) from 5-33 days. 50% of surviving embryos hatched at 30 days. Decrease Na^+ embryonic content and associated with ionoregulatory disturbance.	(Guadagnolo et al., 2001)

Cd ($CdCl_2$)	0.01, 0.02, 0.03, 0.04, 0.10 and 1.00 ppm / eggs to 12-14 days	Java medaka *Oryzias javaniecus*	At 0.01 – 0.10 ppm, death due to developmental impairments, newly hatched larvae showed mortality about 10 – 37%. At 1.0 ppm immediate development arrest. At 0.01 – 0.05 ppm significant premature hatching observed.	(Ismail and Yusof, 2011)
Cd	100 µg L^{-1} / eggs – larval stage	*Leuciscus idus L*.	Reduction in hatching. Teratic effects (bent tail).	(Witeska et al., 2014)
Cd	0.01, 0.02, 0.03, 0.04, 0.10 and 1.00 ppm Cd as $CdCl_2$ / 12-14 days.	Java medaka *Oryzias javaniecus*	At 0.01 – 0.10 ppm death occurred due to developmental impairments. At 1.0 ppm immediate development arrest.	Ismail and Yusof, 2011)
Cd	0, 0.033, 0.1, 0.33 and 1.0 mg L^{-1}, as pulse exposure for 2 hrs at 3, 46, 96 hpf	Rainbow trout *Melanolaenia fluviatilis*	Embryos less tolerant than larvae. Decrease hatching %, spinal deformity.	(Williams and Holdway, 2000)
Cd	1, 2, 4, 8, 16 and 32 mg L^{-1}/ 3-48 hpf	Red sea bream *Pagrus major*	Embryos more sensitive than larvae. At ≥ 0.8 mg L^{-1} delay hatching time, high mortality, and morphological abnormalities. At high concentrations, heart beat and yolk absorption were significantly inhibited.	(Cao et al., 2009)
Cd	0, 0.05, 0.25 and 2.50 µg L^{-1}/ 4 hpf - 56 dpf	Rainbow trout *Oncorhynchus mykiss*	At 0.05 and 0.25 µg L^{-1} premature hatching. At 2.50 µg L^{-1} delay hatching. Larval growth retardation was concentration dependent.	(Lizardo-Daudt and Kennedy, 2008)

Cd^{2+}	1, 3 and 10 µmol / fertilization- 96 hpf	Zebra fish *Danio rerio*	At 10 µM, significant decrease in hatching, Crooked body was seen. Larval swimming speed not affected. Increased larval MDA, and decreased GSH significantly. At 1 and 3 µM SOD decreased. At 1, 3 and 10 µM GPx significantly decreased. At 10 µM GST significantly increased. Larval mRNA of tumour necrosis factor α (TNIFα), interleukin-6 (IL-6), and interleukin-1β (IL-1β) increased significantly.	(Jin et al., 2015)
Cd^{+} Cu sediment	5 g dw sediment Pre – blastula – 10 dpf	Japanese medaka *Oryzias latipes*	Increased heart rate, abnormal spinal column, cardiovascular and morphological abnormalities in hatched larvae, DNA damage. Abnormal heart position looping.	(Barjhoux et al., 2012)
CdSe QDs	0.05, 0.15, 0.45, 1.35, 4.05 and 12.15 mg L^{-1} of MPA-CdSe / 6 – 72 hpf	Zebrafish *Danio rerio*	Toxicity increased as concentrations increased: increased mortality, decreased hatching, and increased malformation (oosperm coagulation, eye spot and melanin developmental inhibition, pericardial edema, bent spin and tail without extension and decreased larval swimming speed.	(Zhang et al., 2012b)
CdTe QDs + Cu^{2+}	6 mg l^{-1} of TGA-CdTe + 0, 0.1, 10, and 100 µg L^{-1} of Cu^{2+} / 6 – 120 hpf	Zebrafish *Danio rerio*	Increased mortality and decreased hatching as Cu concentration increased. Malformation (decrease melanin, pericardial edema, vitelline cyst, bent of spin and tail and decrease somite).	(Zhang et al., 2013)
CdTe QDs	1, 25, 50, 100, 200 and 300 nmol / 6 - 96 hpf	Zebrafish *Danio rerio*	As concentration increased, mortality increased, low hatching success, decreased heart rate at 48 h after exposure, decreased body length, and reduced larval swimming speed.	(Zhang et al., 2012a)

Cr^{6+}	0, 21, 49, 75, 93, 116 and 120 mg L^{-1} / for 96 hrs	Zebrafish *Danio rerio*	Embryos survival and hatchability not affected. At 93 and 120 mg L^{-1}, and at 72 hpf: increased larval mortality, some larvae showed general malformation (e.g., under size, and bowing of vertebral column). At 96 hpf larvae presented behavioural disturbance (difficulty to keep in a normal up right position in water).	(Domingues et al., 2010)
Cr^{6+}	3, 10, and 30 µmol / fertilization- 96 hpf	Zebrafish *Danio rerio*	Non- significant decrease in hatching. Larval swimming speed decrease as Cr concentration increased. Larval MDA increased, and GSH decreased significantly. At 10 and 30 µM, SOD increased, GPX decreased significantly. At 10 µM GST increased significantly. Larval mRNA of tumour necrosis factor α (TNIFα), interleukin-6 (IL-6), and interleukin-1β (IL-1β) increased significantly.	(Jin et al., 2015)
Cu ($CuSO_4.5H_2O$)	50 – 1000 µg L^{-1} Fertilization- 72 hpf	Zebrafish *Danio rerio*	Mortality increased as Cu concentration increased. At higher concentrations: higher mortality occurred between 5 - 24 hpf, decreased hatching, faster heart rate, and slow development of yolk sac.	(Johnson et al., 2007)
Cu ($CuSO_4.5H_2O$)	0, 1, 5, 10, 25, 100, 250 and 500 µmol	Zebrafish *Danio rerio*	Mortality increased as Cu concentration increased. Gene expression changes like Heat shock proteins (HSPs) and enhanced green fluorescent protein (HGFP).	(Hernandez et al., 2011)
Cu ($CuSO_4.5H_2O$)	0.008, 0.015, 0.03, 0.06, 0.13, 0.25, 0.5, 1.0, 2.0, 4.0 and 8.0 mg L^{-1}/ egg – 96 hpf	Rainbow trout *Oncorhynchus mykiss*	At 5.66 mg L^{-1} earlier mortality manifested. Lower hatching. At 0.13 mg L^{-1} reduction in heart rate and at 0.03 mg L^{-1} decrease in body weight was started.	(Stasiūnaitė, 2005)

Cu ($CuSO_4.5H_2O$)	0.1, 0.4, 0.7 and 1.0 mg L^{-1}/ gastrulation stage – 240 hpf	Goldfish *Carassius auratus*	At 0.4 mg L^{-1} significant mortality was seen after 168 hpf. At 0.4, 0.7, and 1.0 mg L^{-1}. Larval deformity was seen as Cu concentration dependent.	(Kong et al., 2013)
Cu ($CuSO_4.5H_2O$)	0.6, 61, 113, 204, 338 and 621 µg L^{-1} / 5-10 to 2 days – hatching.	Fathead minnow *Pimephales promelas Rafinesque*	As copper concentrations increased, embryos survival decreased and larval abnormalities % increased.	(Scudder et al., 1988)
Cu ($CuSO_4.5H_2O$)	100 µg L^{-1} / eggs – larval stage	*Leuciscus idus L*.	Reduction in embryonic survival, delayed hatching, death of the newly hatched larvae, increased body malformation frequency, reduced larval survival. In general embryos were more sensitive than larvae.	(Witeska et al., 2014)
Cu ($CuSO_4.5H_2O$)	0, 0.1, 0.2, 0.4, 0.8 and 1.6 mg l^{-1} (Acute) / 3 hpf – 48 hpf. 0, 0.02, 0.04, 0.06, 0.08, 0.10, and 0.12 mg L^{-1} (Chronic) / 3 hpf – 144 hpf.	Red sea bream *Pagrus major*	Embryos more sensitive than larvae. High mortality, low hatching success and delay hatching time. Heart rate of embryos was not affected by copper exposure. Morphological malformation occurred in embryos and larvae. Reduction in the growth and yolk absorption of the larvae. At ≥ 0.08 mg L^{-1}, decreased heart rate was seen in newly hatched larvae.	(Cao et al., 2010a)
Hg $HgCl_2$	0.01, 0.02, 0.03, 0.04, 0.10 and 1.00 ppm / eggs to 12-14 days	Java medaka *Oryzias javaniecus*	At 0.01 – 0.02 ppm, embryos are very sensitive, while at 0.03 – 0.05 ppm are slightly tolerant, and become sensitive again at 0.1 – 1.0 ppm. At 0.01 – 0.10 ppm, death occurred due to developmental impairments, newly hatched larvae showed mortality about 28.5 – 50.0%. Significant premature hatching was observed.	(Ismail and Yusof, 2011)

Hg (meHg)	2, 5 and 10 mg L^{-1}	Mummichog *fundulus heteroclitus*	Subtle functional impairment (behavioural teratology).Retardation of neurological development.	(Weis and Weis, 1995)
Hg (CH_3HgCl_2)	20 and 30 µg L^{-1} 8,16, 32 h, and continuously – hatching	Zebrafish *Danio rerio*	At 18 – 20 hpf morphological defect as concentration dependent, abnormalities in the median fine fold and flexure of the posterior region of the tail.	(Samson and Shenker, 2000)
Ni	0, 4.2, 7.0, 17.5, 34.3 and 73.9 mg L^{-1} (acute). Fertilization – 96 h. 1, 29, 52, 118, 233, and 466 µg L^{-1} (chronic). Fertilization – 85 days.	Rainbow trout *Oncorhynchus mykiss*	Acute exposure: No effect on plasma Ca, Mg and Na. Chronic exposure: No significant effects on embryo hatching success, larval survival, swim-up, fingerling survival or growth. At acute and chronic exposure, Ni is not an ionoregulatory toxicant.	(Brix et al., 2004)
Pb $Pb(NO_3)_2$	100, 300 and 500 µg L^{-1} 6 hpf – 168 hpf	African catfish *Clarias gariepinus*	ELS are very sensitive to aquatic lead pollutants. Toxicity is dose dependent. Delay in development. At high concentrations (300 and 500 µg L^{-1}) delay hatching. Morphological malformation and histopathological changes.	(Osman et al., 2007)
Pb	0.12, 0.24, 0.48 and 0.96 µmol l^{-1} (pH 5.6 and 7.5)	Common carp *Cyprinus carpio*	At pH 7.5, increased heart rate, decrease body movement, and reduced hatching success. At pH 5.6, reduced hatching success, spinal cord deformation, decreased net Ca^{2+} uptake. Larval mortality increased as concentrations increased.	(Stouthart et al., 1994)

Pd ($Pd Cl_2$)	0, 5, 50, 100, 200, 250, 300, 400 and 450 µg L^{-1}	Zebrafish *Danio rerio*	Toxicity is dose dependent. At 450 µg L^{-1}, decrease survival rate and hatching significantly decrease. At 150 µg L^{-1}, induction of pericardial edema was increased as exposure time increased (48, 72 and 96 hpf).	(Chen et al., 2015)
Zn	0, 0.33, 1.0, 3.3, 10 and 33.33 mg L^{-1} as pulse exposure for 2 hrs at 3, 46, and 96 hpf	Rainbow trout *Melanolaenia fluviatilis*	In general embryos less tolerant than larvae. Decrease hatching %, spinal deformity.	(Williams and Holdway, 2000)
Zn^{2+}	0, 0.1, 0.3, 0.5, 0.7, 1.0, 1.5, 2.0 and 2.5 mg L^{-1} Fertilization – 10 days	Red sea bream *Pagrus major*	Toxicity is Zn concentrations dependent. High mortality 29 – 91 %, increased morphological abnormalities 12 – 77 %, low hatching rate 19 – 78%, and non-significant decreased heart rate.	(Huang et al., 2010)
U	0, 20, 50, 100, 150, 250 and 500 µg L^{-1} Fertilization- 120 hpf.	Zebrafish *Danio rerio*	High mortality of pro-larval stage, delay hatching was started at 250 µg L^{-1}, and growth retardation.	(Bourrachot et al., 2008)

1.7. Biochemical effects of trace metal exposure

1.7.1. Effect of trace metals on Na^+K^+-ATPase

ATPases (Na^+K^+, Mg^{2+}, Ca^{2+}) are groups of ubiquitous integral membrane bound enzymes. They are necessary for the transport of ions through the plasma membrane to achieve several intracellular functions such as regulation of cellular volume, osmotic pressure and permeability of the membrane (Reddy and Philip, 1992). According to their sensitivity, they are considered as biochemical biomarkers for metal toxicity (Yadwad et al., 1990). The dysfunction of the osmoregulatory system due to the interaction of the ATP enzymes with the pollutants have pointed to the importance of ATPase assays as an earlier index of the osmoregulatory disturbances (Stagg et al., 1992; Sancho et al., 2003).

Among these enzymes, the sodium pump (Na^+K^+-ATPase) is a ubiquitous integral membrane-bound enzyme. It is a highly cysteine-rich protein and is composed of two subunits α and β; their presence is necessary for the normal function of the enzyme (Skou and Esmann, 1992; Lingrel and Kuntzweiler, 1994; Scheiner-Bobis, 2002; Lodish et al., 2000) (Figure 1.2). Fish and other aquatic organisms, in order to survive; required to maintain their osmotic pressure higher than that of the surrounding environment (Brooks and Lloyd Mills, 2003). Na^+K^+-ATPase plays an important role to maintain osmoregulation and electrolyte balance by maintaining high Na^+ and K^+ gradient across the plasma membrane (Therien and Blostein, 2000). Normally, Na^+K^+-ATPase utilizes the energy released from the hydrolysis of ATP to ADP (dephosphorylating) to export three Na^+ ions from the cell to the extracellular fluid (ECF) in exchange of two K^+ ions from ECF entering the cell. This contributes to the normal resting membrane potential of animal cells by maintaining the electrochemical gradient across the cell membrane. It also plays a particular role in transporting metal cations across the epithelial cells and is necessary for the regulation of cell volume, cellular pH control, and maintaining free Ca^{2+} concentrations via secondary transporters such as Na^+/Ca^{2+} exchangers (Lamb, 1990; Skou and Esmann, 1992; Li et al., 1996; Handy et al., 2002; Morth et al.,

2011). (Table 1.3) summarized the impairment effects of copper and silver on the activity of Na^+K^+-ATPase.

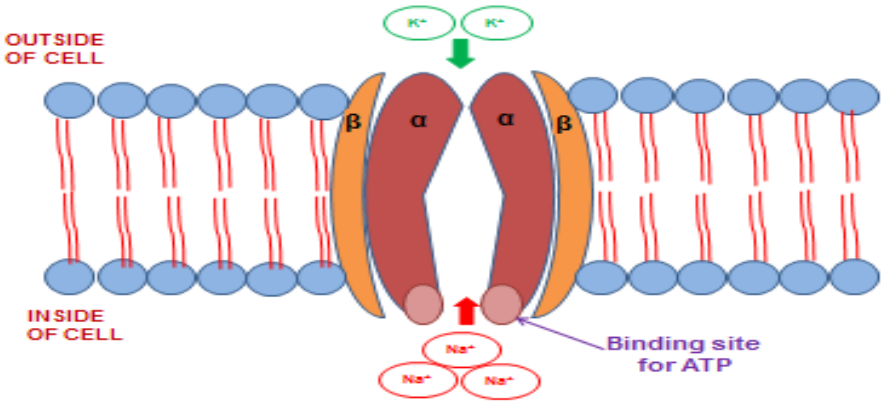

Figure 1.2 Structure of Na^+K^+-ATPase molecule showing the arrangement of α and β subunits and ATP binding site.

1.7.1.1. Effect of copper on Na^+K^+-ATPase activity

Alteration in membrane potential due to the inhibition of the enzyme activity by Cu results in alteration in the electrochemical gradient for Cu uptake from the medium (Handy et al., 2002). Free Cu ions have high affinity to bind covalently with –SH groups of the α subunit of the Na^+K^+-ATPase molecule, which leads to conformational changes in the protein molecule (Kone et al., 1990). On the other hand, competition between Cu with Mg^{2+} on the Mg^{2+} binding site of the Na^+K^+-ATPase molecule causes inhibition of the enzyme activity as a result of the prevention of the hydrolysis of ATP (Li et al., 1996). The chloride cells (ionocytes) in the gills are considered as the major location of the Na^+K^+-ATPase and appear to play an important role in the transepithelial uptake of Na^+. Increased apoptosis and necrosis of the chloride cells in the gill of Mozambique tilapia (*Oreochromis mossambicus*) as copper concentrations increased. The apoptosis is concomitant with decrease in Na^+K^+-ATPase activity and plasma Na^+ concentration (Pelgrom et al., 1995; Li et al., 1998b). Moreover, the early inhibition of Na^+K^+-ATPase activity as a result of gill damage by Cu exposure was documented in rainbow trout (*Oncorhynchus mykiss*) by Shaw et al. (2012), in *Oreochromis niloticus* by Monteiro et al. (2005), and in the amphipod (*Gammarus pulex*) by Brooks and Lloyd Mills (2003). The effect of Cu as an inhibitor of Na^+K^+-ATPase activity and OS inducer was studied by Hoyle et al. (2007) in the intestine as another osmoregulatory organ and also in African walking catfish (*Clarias gariepinus*) gill when fed with dietary copper (1500 mg kg^{-1} dw feed). Transient disturbance of electrolytes, Na^+K^+-ATPase activity, and an increase in total GSH was observed being concomitant with increased TBARS as a marker of lipid peroxidation of the cell membrane of the gill and intestine.

1.7.1.2. Effect of silver on Na^+K^+-ATPase activity

In fish and other aquatic animals, Na^+K^+-ATPase plays an important role in active transport of Na^+ and Cl^- from the aquatic environment to the ECF (Flik et al., 1997; Castilho et al., 2001). Many studies consider Na^+K^+-ATPase molecules located at the basolateral membrane of gill epithelium as the main site of Ag toxicity (Morgan et

al., 1997; Bury and Wood, 1999). As gills are considered as negatively charged ligands, they are targets for positively charged silver ion (Ag^+); thereby the toxicity of silver depends on the saturation of the toxic sites on the biotic ligand (gill) by free silver ions (McGeer et al., 2000a). The interaction between Ag^+ and –SH group leads to conformational changes in the protein (enzyme) molecules leading to impairment of the Na^+K^+-ATPase activity (Kone et al., 1990). On the other hand, silver ion can impair the Na^+K^+-ATPase activity through the competition with Mg^{2+} at the Mg^{2+} binding sites on the cytoplasmic site of α subunit of Na^+K^+-ATPase molecules; in this way, preventing the hydrolysis of ATP is a necessary step for the activation of the enzyme and such impairment causes inhibition in the transport of Na^+ and Cl^- (Hogstrand and Wood, 1998; Wood et al., 1999).

Inhibition of the branchial Na^+K^+-ATPase activity and ion transport as a result of the acute and chronic exposure of silver nitrate ($AgNO_3$) has been studied by many researchers in different aquatic animals. Disturbance of branchial ionoregulation as a result of branchial enzyme inhibition and ion transport was seen during acute silver exposure of rainbow trout (*Oncorhynchus mykiss*) (Morgan et al., 1997). Acute exposure causes complete inhibition of Na^+K^+-ATPase activity in comparison to non-significant inhibition of sub-lethal exposure in major carp (*Cirrhinus mirgala*) (Sathya et al., 2012). Significant changes in Na^+K^+-ATPase activity and intracellular ion concentrations were also documented in marine shrimp (*Penaeus duoraum*) during acute $AgNO_3$ exposure (Bianchini et al., 2005). *Daphnia magna* showed inhibition of Na^+K^+-ATPase activity concomitant with Na^+ uptake inhibition during chronic $AgNO_3$ exposure (Bianchini and Wood, 2002) and acute $AgNO_3$ exposure (Bianchini and Wood, 2003).

Table 1. 3 Effect of Ag and Cu on Na^+K^+-ATPase activity in aquatic organisms

Metals	Concentration/ Time	Species	Effect or Response	Reference
Ag ($AgNO_3$)	0.1 and 10 µg L^{-1} with and without DOC for 44 day	Rainbow trout *Oncorhynchus mykiss*	At 0.1 µg L^{-1} + DOC no effect on ionoregulation. At 23 days of exposure, Na^+K^+-ATPase activity increased at 10 µg L^{-1} + DOC.	(Brauner and Wood, 2002a)
Ag ($AgNO_3$)	5 µg L^{-1} for 21 days	*Daphnia magna*	In chronic exposure Na^+K^+-ATPase activity increased 60 % in whole body.	(Bianchini and Wood, 2002)
Ag ($AgNO_3$)	0.5 µg L^{-1} for 48 h	Daphnids *Daphnia magna*	Na^+K^+-ATPase inhibition related to Ag accumulation in whole body.	(Bianchini and Wood, 2003)
Ag ($AgNO_3$)	1 and 109 µg L^{-1} for 48 h	Shrimp *Penaeus duorarum*	In chronic exposure, 81% significant inhibition in whole body Na uptake, despite Na^+K^+-ATPase activity. Increased 60 in whole body.	(Bianchini et al., 2005)
Ag ($AgNO_3$)	Sublethal 0.0107 mg L^{-1} Acute toxicity 0.107 mg L^{-1} for 96 h	Indian major carp *Cirrhinus mrigala*	In Sublethal exposure, significant decrease in Na^+K^+-ATPase activity. In acute exposure, Branchial Na^+K^+-ATPase inhibited approximately 44% after 96 h of exposure.	(Sathya et al., 2012)
Cu ($CuCl_2$)	0.6 µmol L^{-1} for 1, 4 and 7 days	Mussels *Mytilus galloprovincialis*	At 4 days of exposure, 60 - 70% inhibition of Na^+K^+-ATPase activity in gill.	(Viarengo et al., 1996)

Cu $CuSO_4.5H_2O$	3.2 µmolL^{-1} for 28 days	Mozambique Tilapia *Oreochromis mossambicus*	Decrease in gill Na^+K^+-ATPase activity.	(Li et al., 1998a)
Cu $Cu(NO_3)_2.2H_2O$	Cu 1.9 µmol L^{-1} alone or with pre- treated elevating plasma cortisol.	Common carp *Cyprinus carpio*	Increased plasma cortisol induced increase gill Na^+K^+-ATPase. Decreased plasma cortisol decrease gill Na^+K^+-ATPase activity.	(De Boeck et al., 2001a)
Cu $CuCl_2.2H_2O$	25 to 100 µg L^{-1} for 25 h.	Amphipod *Gammarus pulex*	Reduction in gill Na^+K^+-ATPase activity started at 10 µg L^{-1} increased as dose increased.	(Brooks and Lloyd Mills, 2003)
Cu $Cu(CO_3)_2$	12.8 and 55.2 µmol L^{-1} for 30 days	Gulftood fish *Opsanus beta*	No inhibition of gill and intestine Na^+K^+-ATPase activity.	(Grosell et al., 2004)
Cu $CuSO_4$	40 and 400 µg L^{-1} for 21 days	*Oreochromis niloticus*	Na^+K^+-ATPase activity decreased 5% at 40 µg L-1, and 70% at 400 µg L-1. Decreased was time and concentration dependent.	(Monteiro et al., 2005)
Cu $CuCl_2.2H_2O$	100 µg L^{-1} for 7 days	*Asellus aquaticus* and *Dreissena polymorpha*	Non-significant inhibition of Na^+K^+-ATPase activity.	(Bouskill et al., 2006)
Cu $CuSO_4.5H_2O$	1500 mg k^{-1} dw feed for 30 days	African Walking cat fish *Clarias gariepinus*	Transient disturbances in gill electrolytes and Na^+K^+-ATPase activity.	(Hoyle et al., 2007)

Cu $CuSO_4.5H_2O$	20, 100 µg L^{-1} for 10 days	Juvenile rainbow trout *Oncorhynchus mykiss*	A 6 fold decrease in branchial Na^+K^+-ATPase activity. Significant decrease in brain and intestine Na^+K^+-ATPase activity.	(Shaw et al., 2012)
Cu $CuSO_4$	14.6 µmol $L^{-1}$for 6 hr. after salinity to 2.5, 10.2 and 18.5 ppt (salinity group)	Sheepshead *Cyprinodon variegatus*	Significant decrease in plasma Na^+K^+-ATPase and higher branchial Na^+K^+-ATPase	(Adeyemi et al., 2012)
Cd, Cu, Zn, Pb	5, 10, 20 µmol L^{-1}µmol for 14 days	*Oreochromis niloticus*	Inhibition of gill and intestine Na^+K^+-ATPase activity by all metals, except 20 µM Pb causes increase in enzyme activity.	(Atli and Canli, 2007)

1.8. Role of metallothionein and glutathione in metal homeostasis

Inductions of antioxidants such as GSH addition to metallothionein are all considered as means of metal detoxification (Cortese-Krott et al., 2009). Reduced GSH is considered as the major source of cellular thiol groups, necessary to protect the appropriate redox reaction and viability of the cell (Atif et al., 2005). In contrast, metallothionein, ceruloplasmin, and ferritin are metal-binding proteins that play an important role in transport, metabolism, and homeostasis of essential trace metals as well as detoxification of toxic metals (Roesijadi, 1996).

1.8.1. Metallothionein

Metallothioneins are inducible cytoplasmic non-enzymatic proteins, with low molecular weight (6–7 kDa). They are small intracellular protein molecules with high levels of sulfhydryl groups from the amino acid cysteine and which have the ability to bind Zn^{2+} and other transition metals. The high cysteine content enables Mt to bind the toxic metals ions (Roesijadi, 1996; Kurasaki et al., 2000; Carpenè et al., 2007). Mt is a detoxifying agent involved in the detoxification of excess amount of essential and non-essential transition metals and also regarded as a metal store playing a major role in homeostasis and controlling the replenishing Zn^{2+} ions to the enzymes and other metabolic pathways and binding Cu during toxic excess (Roesijadi, 1996; Amiard et al., 2006).

Copper is one of the transition metals that has an affinity to bind with the thiol-containing molecules, such as Mt, GSH and ceruloplasmin (Pandey et al., 2001), which play a recognizable role in copper metabolism (Brouwer et al., 1989; Ferreira et al., 1993; Luza and Speisky, 1996; Pandey et al., 2001). Ceruloplasmin is a plasma protein that serves as a transporter of copper in the plasma (Luza and Speisky, 1996). Increased ceruloplasmin levels were seen by

Parvez and Raisuddin (2006) in the plasma of *Channa punctata* as a result of copper pre-exposure.

Although copper ion is an essential cofactor in many enzymes, non-chelated or free copper ions react via the Fenton reaction with oxygen to give rise to ROS, such as superoxide radical, hydrogen peroxide, and hydroxyl radical, thereby inducing an increase in Mt synthesis (Ferreira et al., 1993).

Many studies have highlighted the role of copper to induce Mt synthesis. According to Berntssen et al. (1999), increased intestinal Mt concomitant with increased accumulation of copper was seen in Atlantic salmon (*Salmo salar*) when fed dietary Cu concentrations of 35 and 700 mg kg^{-1} for 4 weeks. While exposure of freshwater fish *Oreochromis nitoticus* to 0, 5, 10, and 20 µM Cu for 14 days showed no alteration on GSH and Mt levels in the blood, muscles, and gill. Gill showed increased copper accumulation, whereas only the liver showed an increase in Mt and GSH (Atli and Canli, 2008). In addition, Mt mRNA increased in the gill epithelium of rainbow trout (*Oncorhynchus mykiss*) as a result of gill epithelium hypoxia during the exposure to a concentration 1.65 µM Cu for 24 h (Heerden et al., 2004). Chloride cells are considered as the target of Cu and cortisol, and increased Mt synthesis plays an important role to protect the cells. Dang et al. (2000) observed decrease in the number of glucocorticoid receptor-immunoreactive (GR-ir) cells in the gill epithelium concomitant with increased metallothionein-immunoreactive cells (MT-ir) in the branchial epithelium, and chloride cells of rainbow trout exposed to 2.4 µM waterborne copper; revealing that chloride cells are a place of Mt expression. In addition, differences in fish species may play a role in the synthesis of Mt during the exposure to Cu.

High affinity of silver ions was shown for binding Mt sulfhydryl groups by displacing Zn^{2+}; thus, increased Mt levels lead to scavenging the accumulation of Ag and limiting cell damage (Nielson et al., 1985). Hogstrand et al. (1996a) reported that Ag is a potent inducer of Mt; it was seen that Mt levels in liver and gills of rainbow trout increased as exposure to Ag concentration increased. The

highest level of Mt was observed in liver concomitant with highest accumulation of silver, whereas Wood et al. (1996a) reported that there was no induction of liver Mt, although there was increased Ag accumulation in the liver during exposure of adult rainbow trout to $AgNO_3$ for 6 days. Mt induction in the liver, gills, and kidney occurred in response to the accumulation of Ag in these organs of the adult rainbow trout exposed to silver thiosulfate [$Ag(S_2O_3)_n^-$] (Wood et al., 1996b).

1.8.2. Glutathione

GSH is a major cellular, low molecular-mass thiol compound. It is a tripeptide of glutamate, cysteine, and glycine (L- ɣ- glutamyl-L-cysteinyl-glycine). The presence of ɣ-peptide bond between glutamate and cysteine prevents the degradation of GSH by aminopeptidases. It is found in eukaryotic and prokaryotic cells (Anderson, 1998; Sies, 1999) and participates as a coenzyme in several cellular biochemical functions, such as transport and metabolism of amino acids and maintenance of thiol groups of proteins and low molecular compounds like cysteine and coenzyme A. Moreover, GSH protects against the oxidative damage caused by ROS that is generated during normal metabolism (Anderson, 1998; Peña et al., 2000). GSH as a thiol-containing antioxidant is capable of chelating and detoxifying metals soon when they enter the cell (Fukino et al., 1986). The reduced form of glutathione (reduced GSH), found mainly in the cells as a scavenger of free radicals to maintain the thiol-disulfide status, plays a role in protecting the cell against the oxidizing molecules and harmful xenobiotics such as metals (Peña et al., 2000). The presence of –SH groups in reduced GSH and oxidized glutathione (GSSG) increases the affinity of GSH to bind with the heavy metal ions (Kovářová and Svobodová, 2009). Increased and decreased GSH content in the tissues of fish is affected by the specific response of the organ to the metal exposure (Peña et al., 2000; Sayeed et al., 2003).

GSH has strong affinity for copper ions. The interaction between GSH and copper ions results in a stable complex Cu-GSH as a resident and major

storage protein of copper ions. Thereby, Cu-GSH complex efficiently acts as a donor of Cu ion to Cu/Zn SOD (Ferreira et al., 1993). The response of GSH for the heavy metals was studied by Eroglu et al. (2014) in the liver of fresh water fish (*Oreochromis niloticus*) exposed to 1 µg mL^{-1} individually to Cu, Cd, Cr, Pb, and Zn for 1, 7, and 14 days. It was seen that total GSH levels and GSH/GSSG ratio significantly decreased at 7 days of exposure to metals. In addition, it was reported that copper has a protective effect against a variety of chemicals in mammals. The pre-exposure to copper before the exposure to chemicals offers a protection against the generation of OS (Pandey et al., 2001). According to Parvez and Raisuddin (2006) acclimatization of *Channa punctata* fish with 10 ppb (1 µg L^{-1}) Cu for 4 weeks before exposure to 0.75 µg L^{-1} deltamethrin for 48 h, showed significant reduction in liver GSH levels concomitant with lowered total thiol levels and significant increase in non-protein thiol levels, when compared with deltamethrin-exposed groups.

Previous studies reported GSH depletion in medaka (*Oryzias latipes*) embryos exposed to AgNPs (Wu and Zhou, 2012), whereas increased GSH levels were observed by Choi et al. (2010) due to the induction of GSH-synthesizing enzymes as a response of OS in adult zebrafish exposed to AgNPs.

1.9. Embryonic development / organogenesis

1.9.1. Formation and development of the vertebrate heart

Organogenesis in vertebrates is briefly defined as the formation and development of the organs during embryonic development stages, which is completed before birth or before hatching from one of the embryonic germ layers: ectoderm, mesoderm, or endoderm. During the stages of development, groups of specified and selected cells differentiate to form specific organs characterized by their specific functions (Thisse and Zon, 2002). Expression of specific genes is required during the development of embryos for the

development and evolution of the organs as well as to modify their function (Thisse and Zon, 2002).

1.9.2. Development of zebrafish embryo

A zebrafish embryo is characterized by rapid development of a small fertilized egg (0.7 mm in diameter) compared with other vertebrate animals (Kimmel et al., 1995; Yang et al., 2009). Embryo development is divided into seven periods, which are defined as follows, zygote period (0–0.75 hpf), cleavage period (0.75–2.25 hpf), blastula period (2.25–5.25 hpf), gastrula period (5.25–10.33 hpf), segmentation period (10.33–24 hpf), pharyngula period (24–48 hpf), hatching period (48–72 hpf) and larval period (72 hpf–30 days) (Kimmel et al., 1995). Complete segmentation (24 hpf) is recognized by the initiation of morphogenic movements, complete somitogenesis and the presence of rudiments of many organs (organogenesis). The larvae start their feeding after 5 days post fertilization, an indication that most organs have reached their functional state (Kimmel et al., 1995; Yang et al., 2009).

1.9.3. Formation and development of zebrafish heart

The formation of the heart is a complex morphogenic process, and it is the first organ that forms in vertebrate embryos (Glickman and Yelon, 2002; Targoff et al., 2008). It originates from a group of cells in the anterior lateral plate of embryonic mesoderm (Zaffran and Frasch, 2002; Brand, 2003). Anatomically, the mammalian heart is characterized by four chambers, and in fish, the heart consists of four chambers connected in series, sinus venous, atrium, ventricle and the elastic bulbus arteriosus that converts in teleosts to the contractile conus arteriosus later on (Farrell and Jones, 1992; Hu et al., 2000). The heart contains a special outflow tract and valves that control the direction of blood flow (Fishman and Olson, 1997).

The zebrafish heart, like other vertebrate hearts, is characterized by the presence of specialized endothelial cells known as endocardium and a mass of

musculature that is resistant to high pressure and regulates the electrical rhythmicity of the heart and form specialized chamber (atrium and ventricle), vascular system, and valves (Chen and Fishman 2000). At 10 hpf of embryonic development, somitogenesis starts and reaches 18 somites around 18 hpf, 26 somites at 22 hpf and 29–30 somites at 24 hpf (Stainier et al., 1993). At 16 hpf, the developing embryo possesses 12–15 somites, the cardiogenic differentiation has initiated, and the cells that are located at the midline gave rise to the ventricle, while the cells that are located laterally initiate the formation of the atrium at 22 hpf when the somitogenesis has reached 26 somites, whereas the formation of the heart tube occurs at 30 hpf (Kimmel et al., 1995; De Pater et al., 2009).

1.9.4. Gene regulation of cardiac development in zebrafish

The development of heart is controlled by the specific timing of expression of particular genes. The gene products direct the developmental processes and cooperate in the formation and development of the heart, through the regulation of characteristic proteins of the cardiomyocyte. These genes or transcription factors are known as the gene family (homeobox genes), which include *Tbx5*, *nkx2.5*, *nkx2.7*, *GATA4* and *SALL4* that are essential to maintain the heart function in postnatal life (McGinnis, 1992; Toko et al., 2002; Sultana et al., 2008). The failure or mutation in these transcription factors initiates many cases of congenital heart diseases (CHD) in humans (Akazawa and Komuro, 2003, 2005; Clark et al., 2006). Although many tinman of *nkx* (*nkx2.3, 2.5, 2.7*) were isolated from zebrafish, only *nkx2.5* and *nkx2.7* initiate myocardial differentiation (Chen and Fishman, 1996) and are responsible for cardiac morphogenesis (Tu et al., 2009). Although the transcription of the *nkx2.7* gene was earlier than the *nkx2.5* gene, both of them were required for cardiac morphogenesis. *nkx2.7* played an important role in cardiomyocyte differentiation and cardiogenesis regulation through the regulation of the expression of other genes such as *tbx5* and *tbx20*, which have a role in the development of the heart during heart tube stage (Tu et al., 2009), whereas *nkx2.5* appeared to have a marked effect in the early stages of embryogenesis to initiate the cardiogenic differentiation that led

to the formation of the heart earlier, especially at an early stage of gastrula period. The *nkx2.5* level was on a gradient that initiated the ventral-marginal cells to be developed into heart (Chen and Fishman, 1996). The expression of the *nkx2.5* genes in early embryonic zebrafish development correlated with the cardiac progenitor's position, which reached the embryonic axis at the 8-somite stage to initiate the formation of bilateral tubes at the 18-somite stage. The bilateral tubes fused together during their migration at the midline and the fusion was completed at the 24-somite stage (Stainier and Fishman, 1992; Chen and Fishman, 1996), whereas in other vertebrates, *nkx2.5* was expressed earlier during embryogenesis. It was expressed at the 10-somite stage and continued to be expressed in the myocardial cells of the adult hearts (Chen and Fishman, 1996).

1. 10. Proteomics analysis

Proteomics is a rapidly developing research area that is used for the identification and quantification of the protein complement of a tissue (Shrader et al., 2003). The term proteome was introduced by Marc Wilkins, who described it as 'all proteins expressed by genome or tissue' (Wilkins, 1997), or alternatively as 'a set of all expressed proteins in the cell, tissue or organism at a certain point in time' (Pennington et al., 1997; Plebani, 2005). Although changes in gene expression is a useful method for the measurement of environmental stress (Blom et al., 1992; Lobenhofer et al., 2001), the identification of mRNA alone does not provide information about protein post translation (Cash, 2002). The term, 'proteomics' represents as a link between genes, proteins and diseases (Liebler, 2002).

Proteins vary in their size and composition, and their analysis is complex especially when the determination of the complete mixture of proteins from a tissue is attempted (Cusick et al., 2005). Mass spectrometry-based proteomics has become a powerful method for the detection and characterization of the protein components (Cravatt et al., 2007). Ecotoxicology is the study of an

interaction between the living organisms, ecosystems, and the pollutants that affect the physiology and behaviour of the organisms (Dowling and Sheehan, 2006). Living organisms respond to environmental changes as well as environmental toxins even in low concentrations by changing their gene and protein expressions (Lemos et al., 2010). The application of proteomics technology as a new method to identify the relationship between the toxic factors and ecotoxicology leads to the generation of what is known as 'ecotoxicoproteomics' as a new tool highlighting the early molecular changes as a response to the environmental toxicants (Lemos et al., 2010). Thereby, the application of proteomics in ecotoxicology may give a clear picture about the classes of some functional proteins, such as metallothionein and GSH transferase (Monsinjon and Knigge, 2007). Proteomics analysis highlights the mechanism of the toxic substances on the protein expression profiles, as in the case of mice exposed to 2,2',4,4,5'-pentabromodiphenyl ether (Alm et al., 2006).

The proteomics analysis of zebrafish embryos at early stage of development is a valuable assay to determine the changes in embryonic protein content (Reintsch and Mandato, 2008). Moreover, the application of embryo proteomics as a sensitive toxicity analysis may assist in the earlier assessment of toxicology through the identification of the specific and global protein during embryogenesis (Link et al., 2006; Tay et al., 2006; Lucitt et al., 2008). Exposure of zebrafish (*Danio rerio*) to perfluorooctane sulfonate (Shi et al., 2008) and methyl parathion (Huang and Huang, 2012) showed alteration (increased and decreased) in the expression of some proteins. The alteration in expressed proteins was also observed in the minnow (*Gobiocypris rarus*) exposed to perfluorooctanoic acid (Wei et al., 2008) and in the *Oryzias latipes* exposed to microcystin (Mezhoud et al., 2008). Up regulation and down regulation of some expressed proteins were observed in the anterior gill of mitten carb (*Eriocheir sinensis*) (Silvestre et al., 2006) and in the gill and digestive system of clam (*Ruditapes decussatus*) exposed to cadmium (Chora et al., 2009).

1.11. Hypothesis

There is lack of studies concerning the sensitivity of zebrafish during the early life stages Increase concentrations of Ag as non-essential and Cu as essential trace elements in the aquatic environment increase the risk of aquatic organisms. The sensitivity of zebrafish at early life stages to Ag and Cu probably affects the survival of embryos by affecting different biochemical and molecular aspects (Figure 1.3). Therefore, the study will try to compare the effect of Ag and Cu on the embryonic electrolytes concentrations, $Na^{+}K^{+}$-ATPase activity, total glutathione and *mt2* and *nkx2.5* expressions.

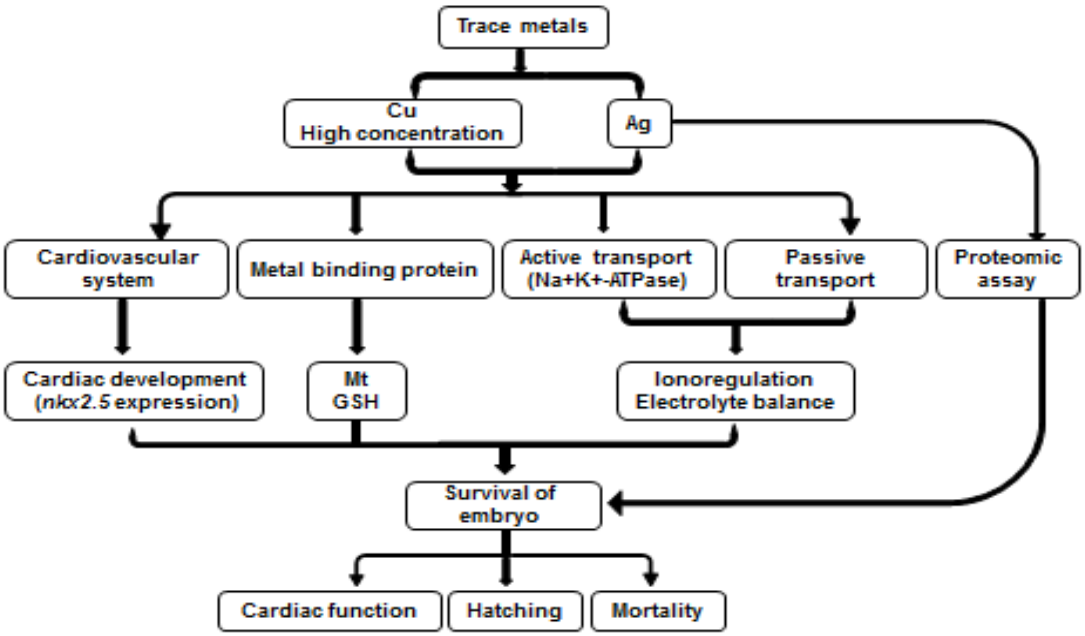

Figure 1.3 Schematic diagram highlights the elements of the hypothesis. The probable effect of Ag and Cu on different biochemical and molecular assays that affects the survival at early life stages of zebrafish.

1.11.1. Aims of the thesis

Overall, the work described in this thesis aimed to investigate the effect of copper and silver exposure via the waterborne route on the survival,

ionoregulation, electrolyte balance, GSH, and expression of *nkx2.5* and *mt2* genes of zebrafish in ELS.

1. The first goal of the study was to determine the most sensitive and vulnerable time and stages of embryonic development that affect the survival, hatching, and cardiac activity by exposing the embryos to waterborne Cu concentrations (Chapters 3 and 4, respectively).
2. Another goal was to assess the toxicity of aqueous Cu in ELS of zebrafish and investigate the ameliorating effect of calcium as the main cation of water hardness to mitigate the Cu toxicity (Chapter 3).
3. In adult fish, ionoregulation and electrolyte balance are the main targets of Cu and Ag and probably are the targets in the ELS that lead to the death of the embryos due to electrolyte imbalance and cardiovascular collapse. Determination of embryonic electrolyte levels as well as the Na^+K^+-ATPase activity will assist to know the effect of Ag and Cu (Chapters 3 and 4, respectively).
4. Another goal of the study was to assess the role of metallothionein and GSH as scavengers and detoxifying agents of free toxic metal ions, such as Cu and Ag ions (Chapters 3 and 4, respectively).
5. Waterborne exposure of Ag and Cu with and without added Ca disturbs the function of cardiovascular system. To assess such an effect, firstly the time and stage of the expression of nkx2.5 gene as one of the essential genes in the differentiation of cardiac myocytes were investigated; secondly, the effects of waterborne Ag and Cu on the expression of the gene were assessed (Chapters 3 and 4, respectively).
6. There have been no studies to investigate the effect of waterborne Ag on the profile of protein expression in developing zebrafish embryos during the 24 hpf. The application of mass spectrometry was used as a recent assay to investigate the protein expression profile in 24 h live embryos (segmentation stage) particularly during the organogenesis stage and their compatibility with biochemical assays.

Chapter 2

General methodology

2. General Methodology

2.1. Experimental animals

Adult zebrafish (*Danio rerio*) aged 0.5 - 1.5 years were obtained from the zebrafish research facility at the University of Plymouth and used to produce embryos for experimentation. The facility was maintained at 28 °C with a 12 h photoperiod. Adult fish were held in glass aquaria with recirculating (10% water replacement per day) de-chlorinated City of Plymouth municipal water. Adult fish were fed *ad libitum* twice daily on flake food (Tetramin) and once daily on *Artemia nauplii*. Adult fish were spawned, and embryos were collected, cleaned of all debris, and used in experiments within 2 h of fertilization.

2.2. Water quality

Water chemistry of "fresh water" was pH 7.53 ± 0.01; temperature, 27.3 ± 0.04 °C; and oxygen saturation, 94.8 ± 0.12%. The concentrations (mean mg L^{-1} ± SE, *n* = 6) of metal ions analysed by Inductively Coupled Plasma Mass Spectrometry (ICP-MS) in the fresh water were Ca^{2+}, 20.91 ± 0.45; K^{+}, 0.69 ± 0.01; Mg^{+}, 0.86 ± 0.01; Na^{+}, 6.32 ± 0.12. Background Cu and Ag^{+} levels in the water were 7.59 ± 0.92 and 0.127 ± 0.070 µg L^{-1}, respectively. The pH, temperature and oxygen saturation were determined using a Hach HQ40d multi reader where it is required.

2.3. Analysis of Trace metals Ag and Cu and electrolytes Ca^{2+}, K^{+}, Na^{+}

The determination of trace metals ions in live and dead zebrafish embryos was performed according to Handy *et al*. (2000). Twenty live embryos and 20 dead embryos were collected in 1.5 mL Eppendorf tubes and rinsed in 0.2 mmol L^{-1} EDTA solution. Samples were dried in an oven (Gallenkamp Oven BS Model

OV- 160) at 70 °C for 24 h. The dried and live embryos were digested in 0.5 ml concentrated nitric acid (69% analytical grad, Fisher Scientific) for 2 h at 60 °C in a water bath, then cooled and diluted to 3 ml with Milli-Q water. Samples of embryos were analysed for embryonic trace metal ions and electrolytes (Ag^{+}, Ca^{2+}, Cu^{2+}, K^{+} and Na^{+}) concentrations by the inductively coupled plasma mass spectrometry (ICP-MS, Thermo Scientific Series 2, Hemel Hempstead, UK). The ICP-MS instrument was calibrated by using mixed matrix-matched standards between (100-100000 µg L^{-1}). The standards were prepared from Aristar® plasma emission grade solutions. During the analysis, the accuracy was checked after every 10 samples by running a blank and standard. Nitric acid (25%) with no metal added was used as a blank to calibrate the ICP-MS instrument.

2.4. Biochemistry

Biochemical analyses such as the $Na^{+}K^{+}$-ATPase, and glutathione are well known as biochemical biomarkers used to investigate the toxicity of trace metals in aquatic organisms (Handy et al., 2003). Embryo samples were collected in 1.5 mL Eppendorf tubes according to the design of the experiment, and stored at -80 °C in order to assay for biochemical analysis, *nkx2.5* and *mt2* gene expression and proteomics analysis.

2.4.1. Preparation of embryo homogenate for biochemical analysis

The first step of biochemical assay performance was included removal of all collected embryos from –80 °C and defrosted on ice. Each sample of collected embryos were homogenised with 400 µl of ice-cold assay buffer containing 20 mmol L^{-1} 4-(2 hydroxyl methyl) piperizine-1-ethane sulfonic acid (HEPES), 300 mmol L^{-1} sucrose and 0.1 mmol L^{-1} EDTA (ethylenediamintetraacetic acid) dissolved in MilliQ water. The buffer was adjusted to pH 7.8 with a solution of Tris HCl). Sonication (Misonix, Microson, Ultrasonic Cell Disrupter, USA), was performed to break down the embryos. To avoid any contamination, the probe

of sonicator was cleaned with 70% ethanol before and after sonication of each sample. The sonicator was adjusted to continuous state on the level 2-3. Each sample was sonicated for 10 s to destroy the embryos. An unbroken visible embryo was sonicated once more. The sonicated samples were stored in ice until the sonication of all samples was completed. Centrifugation was then performed to remove debris from the homogenates, before distribution of aliquots of the embryo homogenates as required for Na^+K^+-ATPase, total glutathione, and protein assays. All the samples were stored at –80 °C until the next step.

2.4.2. Protein assay

The protein assay was performed to determine the changes in the protein concentrations in the embryonic homogenate. A Pierce® BCA Protein Assay Kit was used to investigate the changes in serum protein concentration. Serial standard protein solutions (0 – 2 mg mL^{-1}) were prepared from 2 mg mL^{-1} of bovine serum albumin (BSA) stock albumin standard solution. MilliQ water was used as the diluent. Once embryo samples were defrosted, 10 µl of embryonic homogenate was diluted with 90 µl of MilliQ water (1:10) and mix well in a labelled Eppendorf tube. Ten µl of diluted sample blanks (MQW) and the standards in triplicate were pipetted into appropriate wells. Then 200 µl of working solution (50 part of BCA reagent – A and 1 part of BCA reagent- B mixed in acid washed container) was pipetted into each well. The microplate was covered with tinfoil and incubated for 30 minutes at 37 °C. After incubation the microplate was placed into plate reader (VERSA max tunable microplate reader, Molecular Devices, USA) using the software Softmax® Pro 5) to measure the absorbance at 562 nm. The protein concentrations were calculated by using the standard curve equation.

2.4.3. Total glutathione assay

Total glutathione (i.e. reduced GSH and oxidized GSSG) of the crude homogenate of the embryos was measured according to Smith et al. (2007). Changes in the rate of absorbance were recorded at 412 nm over a period of 15 min with 30 s intervals in a plate reader (VERSA max tunable microplate reader, Molecular Devices, USA) using the software Softmax® Pro 5. Total GSH concentrations in the embryonic homogenates were determined by the reaction of the standard. As the time in kinetic assay is critical, it is important to ensure the rapid addition of the reagents. A multichannel pipette was used to pipette the buffers from the reservoirs. Briefly, 2 mmol L^{-1} reduced GSH (Sigma Aldrich) standard solutions were prepared by dissolving 0.123 g of reduced glutathione in 200 ml MilliQ water. Then 20 µmol L^{-1} of reduced glutathione was prepared by diluting 1 ml of 2 mmol L^{-1} reduced glutathione to 100 ml with MilliQ water. A series of reduced GSH (0 – 20 µmol L^{-1}) standard solutions were prepared by pipetting 0, 0.1, 0.2, 0.3, 0.4 and 0.5 ml of 20 µmol L^{-1} reduced GSH into 1.5 ml Eppendorf tubes containing 0.5, 0.4, 0.3, 0.2, 0.1 and 0 mL of MilliQ water. Twenty microlitres of buffered 10 mmol L^{-1} DTNB (5-5'-*ditbiobis*-(2-nitrobenzoic acid) freshly prepared in assay buffer (100 mmol L^{-1} potassium phosphate, 5 mmol L^{-1} EDTA, pH 7.5), was added to each well of 96 well microplate. Then 20 µl of embryo homogenate, GSH standard and blank (MilliQ water) in triplicate were added to appropriate wells. By using the multichannel pipette 20 µl of 2 U mL^{-1} glutathione reductase (Baker's yeast, G-3664 from Sigma Aldrich, UK) was added to each well, followed by the addition 260 µl of cold assay buffer to each well. After incubation for 1 min, 20 µl of 3.63 mmol L^{-1} NADPH (Melford, UK) was added to each well to initiate the reaction. The changes in the absorbance were used to calculate the total glutathione concentration.

2.4.4. Na^+K^+-ATPase activity assay

The Na^+K^+-ATPase assay was performed according Silva et al. (1977). The assay depends on the liberation of inorganic phosphate (expressed as µmol P_i mg protein^{-1} h^{-1}) from ATP with 40 µl of embryonic homogenate. Twenty mmol L^{-1} potassium phosphate (Sigma Aldrich) standard solutions were prepared by dissolving 0.272 g of potassium phosphate in 100 mL of MilliQ water. Serial dilution of standard (0 – 20 mmol L^{-1}) were prepared from a 20 mmol L^{-1} potassium phosphate standard solution. Forty microlitres of embryonic homogenate and 40 µl of standard and blank in six replicate were placed in small tubes. Three replicate tubes each contained 400 µl of K^+ containing buffer which consisting 100 mmol L^{-1} NaCl, 10 mmol L^{-1} KCl, 5 mmol L^{-1} $MgCl_2$, 1.25 mmol L^{-1} Na_2ATP, 30 mmol L^{-1} HEPES. The media buffer was adjusted to pH 7.4 Four hundred microliters of K^+ free buffer was consisted the same content as the above solution and KCl was replaced by the addition of 1.0 mmol L^{-1} ouabain. (The free K^+ medium was stored in dark bottle to avoid induced oxidation by light).

Samples were allowed to incubate at 37 °C for 10 min in a water bath. The reaction was stopped by the addition of 1 mL of cold trichloroacetic acid (8.6% w/v of trichloroacetic acid / 100 mL MilliQ water) to each tube, and then followed by the addition of 1 mL of colour reagent containing 9.6% w/v $FeSO_4.6H_2O$, 1.15% w/v ammonium heptamolybdate dissolved in 0.66 M sulphuric acid (H_2SO_4). Twenty minutes at room temperature were required for the development of colour. Spectrophotometer (JENWAY, 7315 Spectrophotometer) at 660 nm was used to measure the optical density against 0 - 20 mmol L^{-1} phosphate standards solutions.

2.4.5. Measurement of embryos

To measure the length of each embryo and the volume of yolk sac, 5 embryos were selected from those embryos stored in 10% formalin for histological sectioning. Embryos were photographed (at 4 × magnification) using a Olympus

SZX7 stereomicroscope. Image J 1.46r was calibrated appropriately at scale (1000 pixel/µm, 4 × magnifications) to measure the length of embryo (mm) and yolk sac volume (mm^3). The volume of yolk sac (Vys) was expressed in mm^3 and calculated using the following formula:

$Vys = \pi/6 \times L \times H^2$

Where π (pi) is 3.14, L = yolk sac length (mm) and H = yolk sac height (mm) (Velasco-Santamaría et al., 2011).

2.5. Gene expression

2.5.1. Preparation of embryo homogenate for gene expression

One hundred viable embryos were collected according to the design of the experiment in 1.5 mL Eppendorf tubes for gene expression. The tubes were placed in a box of crushed ice, and the excess of water was pipetted from the tubes. All the tubes were stored temporarily at –80°C for the next step. Sonication (Misonix, Microson XL, 20 levels, Ultrasonic Cell Disrupter, USA), was used to break down the defrosted embryos, the step was started by the addition of 350 µl of RLT buffer to the defrosted embryos. The Eppendorf tube was placed in a 100 mL glass beaker filled with crushed ice to keep the embryo samples cool during the sonication and to avoid any probable effect of the increase temperature on nucleic acids during the sonication. The sonication was started by cleaning the sonicator probe with 70% ethanol. Then the probe was inserted into the middle of the sample to ensure good sonication. Continuous sonication was performed for an initial 5 s on burst level 2 - 3, and then an additional period of sonication was applied to break the visible unbroken embryos. All the samples were stored immediately in ice until the processing of other samples was completed.

2.5.2. Extraction of total RNA

The extraction of total RNA from zebrafish embryos followed the manufactures protocol (RNA easy mini kit for animal tissue, Qiagen) which was developed in the Centre of Environmental Biotechnology (CEB), University of Tennessee, USA. The extraction was started by pipetting 350 µl of embryo homogenate plus RLT buffer mixture onto a QIA shredder spin column placed in 2 mL collection tube. After centrifugation for 2 min at maximum speed (Micro Centaur, MES, 16200 *g*) the supernatant was transferred to a new 1.5 mL Eppendorf tube and further centrifugation for 3 min was applied for more purification by precipitating the solid materials. The cleared lysate was transferred carefully to a new 1.5 ml Eppendorf tube, and mixed well with 350 µl of 70% ethanol; the mixture (700 µl) was transferred to the RNeasy minicolumn placed in a 2 mL collection tube. The sample centrifuged at maximum speed 16200 *g* for 15 s, the eluted flow-through was discarded, leaving the RNA stuck to the silica gel.

2.5.3. DNA digestion

In order to purify the RNA sample, DNase treatment was used to remove any DNA that causes contamination of the RNA sample. The protocol was started by washing the content of RNeasy minicolumn by pipetting 350 µl buffer RW1 into RNeasy spin column and centrifuged at maximum speed 16200 *g* for 15 s. The flow-through was discarded after centrifugation. Then 80 µl per sample of a mixture of 10 µl of the DNase I stock solution and 70 µl of RDD buffer was pipetted directly onto the RNeasy silica gel membrane of the RNeasy minicolumn. The mini column was placed at room temperature (20 ± 2 °C) for 15 min on the bench top. Then 350 µl of RW1 was added once more to wash the RNA stuck to the silica gel. After centrifugation at maximum speed 16200 *g* for 15 s, the flow-through was discarded. To avoid any contamination the minicolumn was transferred into a new 2 mL collection tube. The next step included two additions of 500 µl of RPE buffer to wash the minicolumn twice. The flow-through was discarded, after centrifugation at a maximum speed

16200 *g* for 15 s. The mini column was centrifuged once more for 2 min at maximum speed 16200 *g* to confirm the dryness of the RNeasy silica-membrane gel. The last step in the extraction of RNA was included the transfer of the column into a new 1.5 mL collection Eppendorf tube, and 30 µl of RNase-free water was pipetted directly onto the RNeasy silica gel membrane. The column was left for 2 min to sit and then centrifuged for one minute at 16200 *g* to elude the RNA. The total concentration of RNA was measured by a Nano Drop spectrophotometer at 260 nm for the maximum absorbance (Nano Drop, ND-1000 Spectrophotometer, Labtech International). The optimum quality value for spectrophotometer of RNA: < 100 ng μl^{-1}, and the acceptable ratio for the absorbance of RNA/DNA 260:280 is 1.9 - 2.2 for the identification of the purity of RNA and to indicate any contamination with proteins, while the absorbance ratio for RNA: protein 260/230 is 1.9 - 2.0. All the RNA elute samples were stored at –80 °C for the next step.

2.5.4. Reverse transcription of RNA to generate cDNA

The initial step of cDNA synthesis was the preparation of the RNA template. The extracted RNA was diluted to 100 ng μl^{-1} total RNA, by pipetting 20 µl of the extracted RNA sample into a 1.5 mL Eppendorf tube, and then an adequate volume of nuclease-free water was added according to the concentration (ng/µl) of the extracted RNA by using the following equations:

Sample concentration/100 = dilution factor

Dilution factor × 20 (extracted RNA volume) = µl (total volume)
Total volume – 20 = µl (water volume to be add to 20 µl of RNA).
All the normalized samples to 100 ng/µl total RNA were kept on ice during the addition of nuclease–free water.

According to the ImProm – II™ Reverse Transcription System, Promega, (Sigma-Aldrich) protocol was used to synthesize cDNA from total RNA enough for triplicate samples for Q-PCR. Eight hundred ng (8 µl) RNA was used to

synthesise cDNA of each sample was pipetted to the sterile nuclease-free thin-walled Bilatec 0.2 ml PCR tube. Sixteen µl of RT-Mix (6.6 µl nuclease-free, 4 µl ImProm-II™ 5X reaction buffer water, 2.4 $MgCl_2$ (25 mm), 1 µl dNtp mix (10 mM each dNTP), 1 µl hexanucleotide primers and 1 µl ImProm-II™ Reverse Transcriptase) were added to each tube. All the tubes were closed and kept on the ice to avoid contamination and evaporation. The synthesis of cDNA was performed by using a thermal cycler machine (GeneAmp® PCR System, 9700, Applied Biosystems). The following procedure was used in the synthesis of the cDNA: annealing temperature at 25 °C for 5 min, extending for 60 min at 42 °C and heat inactivating the transcriptase by incubating all the tubes at 70 °C for 15 min. The cDNA samples were stored at -80 °C until required for q-RT-PCR gene expression analysis.

2.5.5. Quantitative reverse transcriptase PCR (qRT- PCR)

Primers of *nkx2.5*, *mt2* and housekeeping gene (*β actin*) were selected using Primer Blast (National Centre for Biotechnology Information, NCBI) Gen Bank. The primer pairs were used for *nkx2.5*: 5'-AGTTCTCTTCTCTCAGGCGCAGG – 3' (forward) and 5'- TGGCACAGAGATGCGTCTCGGA-3' (reverse), for *Mt2*: '5-CTGCGAATGTGCCAAGACTGGAAC-3' (forward) and 5'-GCGATGCAAAACGCAGACGT-3 (reverse). For housekeeping gene *β actin*: '5-ACACAGCCATGGATGAGGAAATCG-3' (forward) and 5'-TCACTCCCTGATGTCTGGGTCGT-3 (reverse). Primers details are listed in Table 2.1.

The protocol for the quantitative analysis of *nkx2.5*, *mt2* and *β actin* gene expression was followed the procedure of Sigma SYBR® Green Start™ Taq Ready Mix™. Lyophilised primers (Eurofins MWG Operon, Ebensburg, Germany) were reconstituted to 100 µmol L^{-1} with nuclease free water, and mixed with SYBR Green JumpStart Taq Ready Mix to give a final reaction concentration of 375 nmol in 20 µl total volume. Master Mix for each sample was prepared as follows: 12.5 µl SYBR Green JumpStart Taq ReadyMix, 0.75

µl forward primer and 0.75 µl reverse primer. Ninety six well plates (Applied Biosystems™ MicroAmp® Fast 96-well, Thermo fisher Scientific, China) were used for qRT- PCR reaction. The sample was diluted 1:10 and 6 µl of the diluted sample was mixed with 16 µl of Ready Mix. A negative control (20 µl nuclease- free water only) and a no template control (6 µl nuclease free water and 16 µl of Ready Mix) were also prepared. All the target genes samples, negative, no template controls and β actin were done in triplicate. The fluorescence was detected (StepOne Real-Time PCR System, Applied Biosystems) over 40 cycles. The protocol of thermal qPCR reaction included the following stages: Holding stage (incubation period at 94 °C for 2 min) followed by cycling stage (40 cycles), started with denaturation step at 94 °C for 15 s, then annealing of the primers at 55 °C for 1 minute and the extension at 72 °C for 1 min, with a selection of fluorescence. On the same plate the standard curve of cDNA was run for plate normalization. The cycle threshold was set to 25,000 for qRT-PCR runs. The changes in the fold ($2^{-\Delta\Delta Ct}$) were calculated according to the differences between the reference control and the target genes (Henry et al., 2009). Calculation of fold-changes in expression of *nkx2.5* and *Mt2* gene transcripts was conducted after normalization to *β-actin* by the comparative quantification method ($2^{-\Delta\Delta Ct}$) (Henry et al., 2009).

Table 2.1 Zebrafish (*Danio rerio*) gene specific primers (*nkx2.5, mt2* and *β-actin*) reference sequence numbers from NCBI, and product length in base pairs.

Gene	Ref. Seq.	Forward (5'-3')	Reverse (5'-3')	Product No	Annealing temp °C
nkx2.5	NM_131421.1	AGTTCTCTTCTCTCAGGCGCAGG	TGGCACAGAGATGCGTCTCGGA	223	58
β-actin	NM_131031.1	ACACAGCCATGGATGAGGAAATCG	TCACTCCCTGATGTCTGGGTCGT	138	55
Mt2	NM_001131053.2	CTGCGAATGTGCCAAGACTGGAAC	GCGATGCAAAACGCAGACGT	243	58

2.6. Routine statistical analyses

Statistical analyses were conducted with STAGRAPHICS 5.1 (Statistical Graphics Corp., USA). Excel spreadsheet were used to draw the figures. Bartlett`s test was used initially for variance checking. Dependent variables (i.e., the measurement endpoint of the experiment) were modelled according to the independent variables copper and silver concentrations (continuous variable), presence of calcium (added or not added), and whether embryos were dead or alive. When data were normally distributed and variances homogeneous, the relation between independent variables was modelled by two-way ANOVA with the inclusion of the appropriate interaction term. If the interaction term was significant, simple effects of levels of individual independent variables were assessed by one-way ANOVA. A probability value of $p < 0.05$ was considered significant and all results are presented as mean ± standard error (SE).

Chapter 3

Effect of calcium on copper accumulation and toxicity during embryonic development in zebrafish (*Danio rerio*): biochemical and molecular aspects

Effect of calcium on copper accumulation and toxicity during embryonic development in zebrafish (*Danio rerio*): biochemical and molecular aspects

Abstract

Although copper is an essential micronutrient, elevated aqueous concentrations can be toxic to the early life history stages of fishes. This study aimed to determine the stage of zebrafish embryonic development that is most vulnerable to dissolved copper (Cu) toxicity and to investigate effects on cardiac development and embryo biochemistry. An initial experiment identified the lethal concentration of Cu to zebrafish embryos in Plymouth freshwater of around 300 µg L^{-1} in 24 h. Further experiments explored the sub-lethal effects of Cu in normal Plymouth freshwater, and another series determined the effect of adding more Ca to the water (40 mg L^{-1}). Embryos were analysis for metals, Na^+K^+-ATPase activity, total GSH, and changes in target gene expression depending on the experiment. In normal Plymouth fresh water, embryo mortality was Cu concentration-dependent, and the developmental stage at 10 hpf (blastula and gastrula stages) was found to be the most vulnerable to Cu toxicity. A decrease in hatching and increased heart rates was observed in embryos at 36 hpf. In the experiment exploring the modulating effects of added water Ca, an apparent accumulation of Cu was observed in both live and dead embryos, regardless of the water Ca concentration. However, the calcium concentration passively increased with embryonic copper concentration in dead embryos. Na^+ and K^+ concentrations were not affected by Cu exposure or elevated Ca. In normal Plymouth freshwater a 4 fold decrease in Na^+K^+-ATPase activity was seen in live embryos exposed to copper compared to controls, and added water Ca prevented this effect. There were no effects on total glutathione. Expression of *nkx2.5* as one of the essential genes for the formation and development of the heart increased significantly; approximately 10 fold in the presence of Cu plus extra Ca in comparison to the unexposed controls or Cu exposure alone in Plymouth fresh water. Conversely, the expression of *mt2* increased significantly 6 fold compared to the control during Cu exposure in normal Plymouth

freshwater, but not with extra Ca. Overall the data show that Cu toxicity in embryos is consistent with disturbed osmoregulation with induction of *mt2* expression; and added calcium protects against copper toxicity. Ca^{2+} modulates the expression of the *nkx2.5* gene, but only during Cu exposure, although the mechanism requires further investigation.

3.1. Introduction

Copper enters surface waters naturally as well as via anthropogenic activities and is of considerable environmental concern because of its toxicity to aquatic organisms. In surface waters, copper can exist as dissolved ions (e.g., Cu^{2+}) and also in complexes with dissolved organic material and some inorganic anions (Meylan et al., 2004). Although copper is an essential micronutrient used in various enzyme systems (Sorensen, 1991), elevated environmental concentrations have been shown to have a variety of negative effects on fish physiology that include ionoregulatory disturbance (Li et al., 1996; Grosell and Wood, 2002; Handy et al., 2002), damage to the olfactory system (Hansen et al., 1999), and disruption of fish behaviours that are critical for survival (Hernández et al., 2006; Linbo et al., 2009). The toxicity of copper in fish has been investigated for decades (reviews, Taylor et al., 1996; Handy, 2003; Kumande and Wood, 2004; Bury and Handy, 2010) and numerous detailed aspects of the biochemical mechanisms of toxicity have been revealed including some of the various exogenous (e.g., calcium ion concentrations) and endogenous (e.g., expression of metallothionein proteins) factors that influence the toxicology of copper.

Embryonic life stages of fish are especially sensitive to low concentrations of dissolved copper and toxicity appears to vary with the stage of development (Schilling, 2002). Mortality and delayed/decreased hatching are often reported with results varying according to fish species and copper concentration. For example, during the first 24-h post fertilization (hpf) in zebrafish (*Danio rerio*) hatching was delayed and 100% mortality was observed at a concentration of ~1000 µg Cu L^{-1} (Johnson et al., 2007). Similar findings were reported in another study on zebrafish (Hernández et al., 2011), although the lethal Cu concentrations were somewhat lower. In gold fish, *Carassius auratus*, embryo mortality increased with Cu concentration and some deformities of larvae were also reported (Kong et al., 2013).

In addition to mortality, delayed hatching and deformities in embryos of Red sea bream *Pagrus major* reduced heart rate was also reported during exposure to Cu concentrations of 0.08 mg L^{-1} (Cao et al., 2010a). However, both increases and decreases in heart rate have been observed in early life stages of fish. A significant increase in heart rate was noted at 28 hpf in zebrafish embryos with increased concentration of Cu (Johnson et al., 2007); whereas, decreased heart rate was found in common red carp (*Cyprinus carpio*) embryos exposed to 0.05 mg Cu L^{-1} (Stouthart et al., 1996), and no effect of Cu on heart rated was documented in Red sea bream exposed to 0.080 mg L^{-1} (Cao et al., 2010b). Other dissolved metals have also been found to have different effects on heart rate in embryonic fishes. For example, Japanese medaka *Oryzias latipes* embryos had increased heart rate upon exposure to cadmium (Barjhoux et al., 2012), whereas heart rate of Red sea bream was not affected by exposure to Cd (Cao et al., 2009). Exposure to Zn (2.5 mg L^{-1}) did not significantly affect the heart rate of red sea bream embryos, but increased heart rate was observed in larvae at concentrations ≥ 1.0 mg L^{-1} (Huang et al., 2010). Results of the effect of dissolved metals on fish heart development and function have been inconsistent and need further investigation.

The heart is the first organ to develop and function in vertebrate embryos and can be observed in zebrafish as early as 24 hpf (Glickman & Yelon, 2002; Targoff et al., 2008). Cardiac development is controlled by the specific timing and expression of particular genes including *GATA, nkx, MEF2, Tbx* and Hand gene families (McGinnis, 1992). *Nkx2.5* appears to be involved in initiating the differentiation of cardiac cells in the early embryonic stage of development of both mammals and zebrafish (Chen & Fishman, 1996), and changes in the expression of *nkx2.5* genes has been documented in zebrafish exposed to polycyclic aromatic hydrocarbons (PAHs) (Zhang et al., 2012). There has been considerable advancement in understanding of the molecular mechanisms involved in cardiac development in zebrafish, but the influence of metal exposure on these mechanisms has not yet been investigated although Cu homeostasis is implicated in congenital heart defects in humans (Hu et al., 2014).

The toxicity of Cu is reduced by the presence of aqueous calcium and by the induction metal detoxifying proteins (e.g., metallothionein). Increased Ca concentrations as major component of water hardness can help prevent Cu binding and toxicity at the gills (Pagenkopf, 1983), and thus subsequent osmoregulatory disturbances. Sodium homeostasis is often a critical aspect of the aquatic toxicity to fishes (Handy et al., 2003) with concerns about the inhibitory effects of Cu on the Na pump (Li et al., 1996). The Na^+K^+-ATPase is also essential for the embryonic development and function of the heart in zebrafish. It seems that the Na^+ pump α2 isoform is necessary for establishing cardiac laterality, whereas, the α1B isoform is necessary for the extension of the primitive heart tube and cardiomyocyte differentiation (Shu et al., 2003). The interaction of the electrogenic Na^+ pump with the Na^+/Ca^{2+} exchanger is also well-known for regulating the excitation-contraction coupling in mammalian cardiac cells (Vaughan-Jones, 1986), but less is known about the interactions of Cu, Ca^{2+}, and Na^+/K^+-ATPase activity in embryonic fish.

Metallothionein is well-known for its role as an intracellular metal chelator during Cu toxicity in adult fishes, but the situation is less clear in developing embryos. At the early stage of zebrafish embryonic development, and immediately after fertilization, the metallothionein is of maternal origin (i.e., present in the yolk). At the gastrula stage, maternal metallothionein content decreases (Riggio et al., 2003). Exposure to Cd^{2+} during the blastula stage increases metallothionein content in zebrafish embryos (Riggio et al., 2003; Chen et al., 2004), presumably of embryonic origin since exposure of embryos at 8 hpf for 24 h to different metals was reported to increase expression of metallothionein genes: Hg^{2+} (up to 50 fold) > Cd^{2+} (up to 20 fold) > Cu^{2+} and Zn^{2+} (up to 5 fold) (Chan et al., 2006).

The aims of the present study were to investigate the effect of Cu exposure on early developmental stages of zebrafish embryos. The specific objectives were to identify the most vulnerable stage of embryonic development, and evaluate whether Cu exposure affects the Na^+K^+-ATPase activity, electrolyte balance, and as well as the expression of *nxk2.5* and *mt2* gene transcripts during

embryonic development. Then, give the general protective effects of calcium on metal toxicity and its importance in cardiac physiology to determine the influence of calcium additions on the Cu-dependent responses of these parameters.

3.2. Material and method

3.2.1. Experimental design

Experiments were conducted to address the following objectives: (1) to determine the relation between stage of embryonic development and expression of the *nkx2.5* gene transcript; (2) to determine the period of embryonic development most vulnerable to Cu toxicity; and (3) to determine the effect of calcium on Cu toxicity.

3.2.2. *Nkx2.5* gene expression

Two experiments were conducted to establish the relationship between *nkx2.5* gene expression and stage of embryonic development. Each experiment consisted of a batch of embryos which were fertilized at the same time (within 15 min) from multiple pairs of male and female fish. Embryos < 1 hpf were stocked into eight 400-mL glass beakers (170 embryos/beaker) containing 300 mL of fish water and beakers were randomly assigned a time post fertilization (hpf) in which all embryos were sampled for assessment of gene expression. In experiment 1, sampling occurred at 5, 10, 13, 16, 19, 24, 28 and 34 hpf; and in experiment 2, sampling occurred at 10, 13, 16, 24, 28 and 34 hpf.

3.2.3. Effect of copper on early stage of embryonic development

After a range finding experiment to identify lethal and sublethal concentrations of Cu to zebrafishes in Plymouth water, two main series of experiments were conducted to: 1) determine the period of embryonic development most vulnerable to Cu toxicity; and 2) determine the effect of calcium on Cu toxicity.

In the acute toxicity range finding experiment, 25 embryos (age < 1 hpf) were stocked into each beaker (400 mL glass beaker with 300 mL exposure water), in triplicate, and exposed to Cu as $CuSO_4.5H_2O$ (Sigma-Aldrich) at total nominal

Cu concentrations 0, 50, 100, 200, 300, 400, 500 and 1000 µg L^{1}. Embryos were exposed at 28 °C with a 12 h photoperiod, and mortality was recorded at 10 hpf and 24 hpf. Following the range finding experiment, an exposure concentration of 500 µg L^{1} was selected for the second and third experiments.
In the second experiment, embryos were exposed at different points during development to identify the most Cu-sensitive window in development as well as explore physiological effects. The Cu exposure periods were as follows: 1-10 hpf, 10-72 hpf, and 1-72 hpf. In each treatment, 30 fertilized embryos (age < 1 hpf) were stocked in each beaker and each experimental condition (i.e., exposure period) had three beakers (i.e., 90 embryos/treatment). Embryos that were exposed from age 1-10 hpf were transferred to clean beakers and fresh fish water without Cu, whereas, the embryos of the second group were exposed to Cu from 10 to 72 hpf, and the third group of embryos was exposed from 1-72 hpf. At 24 and 36 hpf. Nine embryos were randomly sampled from each beaker for assessment of heart rate (heart beats/min). A binocular microscope was used for counting the heart beats over 60 s.

Some mortality was monitored at 10 and 24 hpf (none occurred after this), while hatching was determined at the end of the experiment (72 hpf) in all treatment groups. Then having identified potentially sensitive times in development, a more detailed trial was conducted to evaluate the effect of sub-lethal copper exposure on cardiac function according to the precise periods of embryonic development. Exposure to 500 µg L^{-1} Cu occurred during the following developmental periods: blastula (2.25-5.25 h), gastrula (5.25-10.33 h), segmentation (10.33-24 h) and pharyngula (embryos exposed between 24-36 h). A control (no copper exposure) and a continuous exposure (500 µg L^{-1}) were used for comparison with exposures during specific developmental periods. Twenty five fertilized embryos < 1 hpf were stocked into six beakers (400 mL glass beaker with 300 mL exposure water), with each beaker representing one stage of embryonic development. Ten embryos were selected randomly at 36 hpf for determination of heart rate (as described above).

3.2.4. Effect of added calcium on copper toxicity

The effect of calcium on Cu toxicity during embryonic development was evaluated in the final series of experiments. Three identical trials were conducted for logistical reasons to obtain enough biological material for the different biochemical measurements. The Cu control in the design was a range of Cu exposures (0, 100, 250, and 500 µg Cu L^{-1}) in water containing normal Ca, and the treatment was the same Cu exposures, but conducted with extra Ca added to the Plymouth freshwater (nominal 40 mg Ca^{2+} L^{-1} with 0, 100, 250, or 500 µg Cu L^{-1}). Each exposure trial was conducted in triplicate (400 mL glass beaker with 300 ml exposure water) with 170 fertilized embryos stocked into each test vessel. In each trial, the exposure was ended at 16 hpf (exposure was from 2-16 hpf). From each beaker in experiment 1 and 2, twenty live and twenty dead embryos were collected for total metals concentrations, and 60 live embryos were collected for assessment of total glutathione, total protein content, and Na^{+}-K^{+}-ATPase activity. In the third experiment, 100 live embryos were collected from each beaker for gene expression analyses. All embryos were stored at -80 °C until they were used for analysis. The background concentration of Cu and Ca in the Plymouth freshwater for the final series of experiments (no added Cu or Ca) was 7.17 ± 0.26 µg L^{-1} and 20.91 ± 0.45 mg L^{-1} respectively (see metal analysis below). The actual concentrations (mean ± SD, N = 6) of Cu in beakers with added copper only (0, 100, 250 and 500 µg L^{-1} Cu, normal Ca), were 7.17 ± 0.26, 73.15 ± 0.73, 199.53 ± 4.81, 428.6 ± 4.12 respectively. In the treatments with added Ca, the total Cu concentrations were 5.90 ± 0.54, 71.34 ± 1.62, 200 ± 3.33, 419.97 ± 3.37 µg L^{-1} for 0 (no added Cu), 100, 250 and 500 µg L^{-1} Cu), respectively. The measured Ca concentrations in the latter were; 20.56 ± 0.39, 20.47 ± 0.10, 20.14 ± 0.18, 20.26 ± 0.08 for copper treatment control, and 55.07 ± 1.8, 55.61 ± 1.01, 56.22 ± 1.21, 55.77 ± 0.79 mg L^{-1} for copper with added calcium.

3.2.5. Trace metal analysis

The determination of trace metals in live and dead zebrafish embryos was performed according to Handy et al. (2000). Twenty live embryos (ww 39.58 ± 0.002 mg) and 20 dead embryos (ww 30.23 ± 0.001 mg) were collected in 1.5 mL Eppendorf tubes, and rinsed in 0.2 mmol L^{-1} EDTA solution. Samples were dried in an oven (Gallenkamp Oven BS Model OV- 160) at 70 °C for 24 h. The average weight of dried live embryos was 38.52 ± 1.78 mg, and of dead embryos was 29.31 ± 0.81 mg. Other steps for trace metal analysis are described in Chapter 2.

3.2.6. Biochemistry

Biochemical analyses were performed exactly as described in Chapter 2.

3.2.7. Gene expression

Preparation of embryos and extraction of RNA were performed as described in Chapter 2.

3.2.8. Statistical analysis

StatGraphics Plus version 5.1 was used to analyse all the data. One way analysis of variance was used to identify the effect of copper with and without added calcium at the end of experiment (16 hpf). Fisher's least significant difference (LSD) test was used to identify differences between the means of treatments. Bartlett's test was used to check the validity of each ANOVA. To check the differences between copper exposure treatment with and without added calcium two way ANOVA was used. Results are presented as mean ± S.E.M. For the calculation of fold-changes in the *nkx2.5* and *mt2* with normalization to β-actin a comparative quantification ($2^{-\Delta\Delta Ct}$) was used (Henry et al., 2009).

3.3. Results

3.3.1. *Nkx2.5* expression

The expression of *nkx2.5* gene transcripts was lowest early in embryonic development at 5 hpf, increased between 10 and 16 hpf, and then appeared to decrease later in development from 18 - 34 hpf (Figure 3.1). Although expression of *nkx2.5* was highly variable (R^2 = 0.49) over embryonic development. The fit of a quadratic equation was non-significant (ANOVA, P > 0.05) and indicated highest expression at 16 hpf.

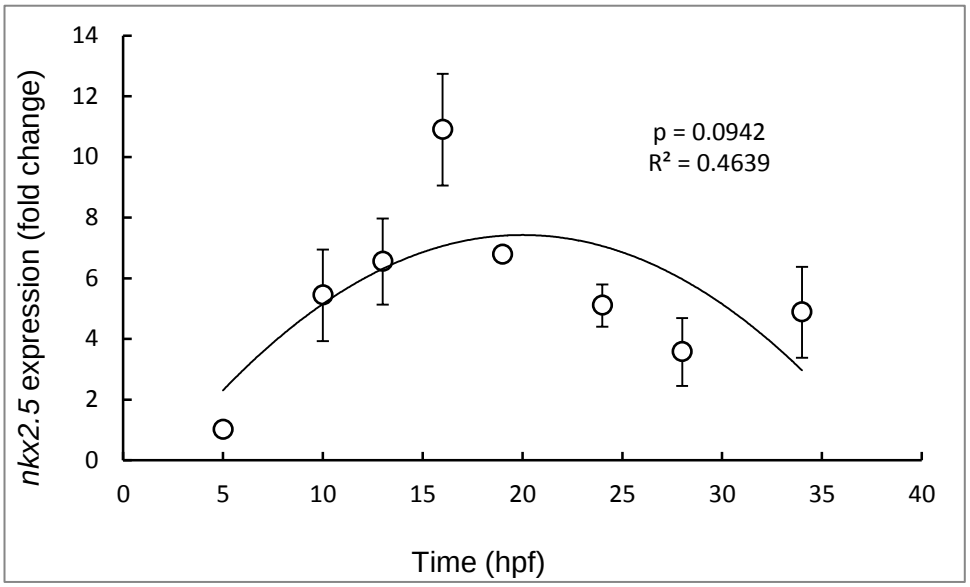

Figure 3.1 Fold change in *nkx2.5* expression relative to stage of zebrafish embryonic development from two experiments (Exp1, Exp2). Embryos were sampled at different times postfertilization and *nkx2.5* expression was assessed by the delta-delta C_t method after normalization to *β-actin*. Each point is the relative change in gene expression for an individual sample that consisted of 100 embryos. Fold change between concentrations that were assessed by one-way ANOVA (p > 0.05).

3.3.2. Stage of embryonic development and Cu toxicity

The mortality of the zebrafish embryos increased with the total Cu concentration in the water and higher cumulative mortality was observed in embryos exposed for 24 h compared to embryos exposed for 10 h (Fig. 3.2). Embryos exposed to 500 µg L^{-1} Cu for first 10 hours of development (1-10 hpf) and the continuous exposure treatment (1- 72 hpf) showed statistical significant increase in mortality (one way ANOVA, $p < 0.05$) in comparison to the unexposed controls (Fig. 3.3A). Mortality in the 1-10 hpf treatment was also different from the 10 – 72 h groups (Fig. 3.3A). No mortalities were observed in any treatment after 24 hpf.

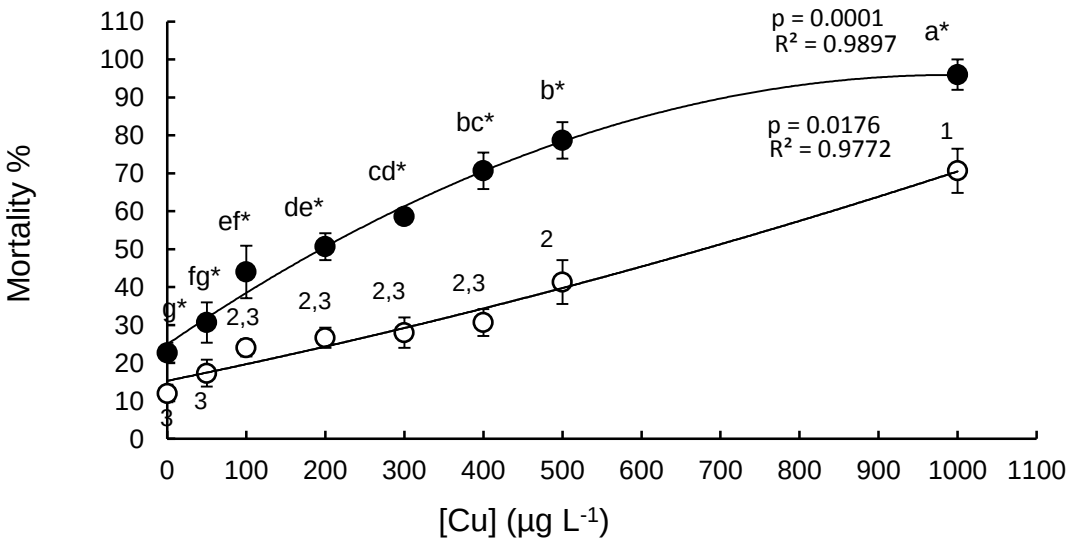

Figure 3.2 Mortality of zebrafish embryos relative to the copper concentration at 10 hpf (open circles) and at 24 hpf (solid circles). Data are means ± standard error, *n* = 3 independent samples per treatment (each sample was a beaker that contained 25 embryos). Different letters and numbers indicate significant differences between concentrations that were assessed by one-way ANOVA ($p < 0.05$). * indicates significant differences (ANOVA, $p < 0.05$) between mortality at 10 and 24 hpf.

Heart rate was also assessed at two critical points in embryonic development – at 36 hpf and at 24 hpf (when the heart should be anatomically formed). The continuous exposure of embryos to the same concentration of copper as above (500 µg L^{-1}) resulted in a significant increase in heart rate (one way ANOVA, $p < 0.05$) at 36 hpf in comparison to the unexposed control and all the other groups (Fig. 3B). In addition, significantly higher heart rates (two way ANOVA, $p < 0.05$) were recorded at 36 hpf in comparison with 24 hpf respectively in all treatments (Fig. 3B).

At the end of the experiment (72 hpf) the hatching success of all the treatment groups were determined (Fig. 3C). At 72 hpf the embryos subject to continuous Cu exposure (1 – 72 hpf) had a lower hatching rate in comparison with the other groups (one way ANOVA, $p < 0.05$, Fig. 3C).

Given the observed changes in heart rate above, a further trial was conducted to determine at what stage of development that Cu most affected heart rate. To establish the window of cardiac sensitivity to Cu, each stage of development was exposed to 500 ug L^{-1} Cu compared to an unexposed control and a continuous Cu exposure treatment (Fig. 4). Then, at 36 hpf, the heart rate was measured in animals from all the treatments. Embryos at the pharyngula stage showed higher heart rates at 36 hpf (ANOVA, $p < 0.05$), while the embryos at the blastula stage showed the lowest heart rate in comparison to other stages of development (Fig. 4).

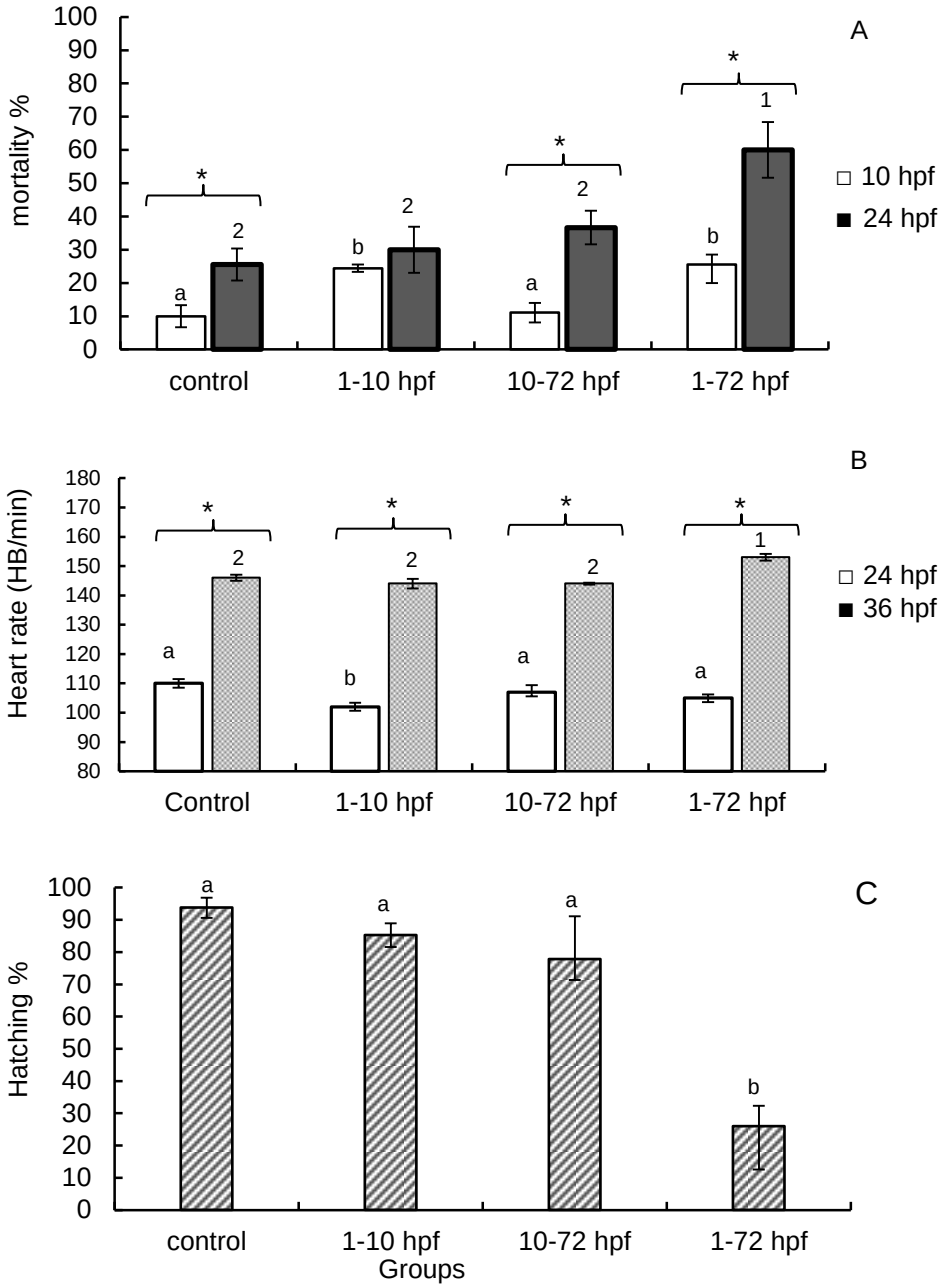

Figure 3.3 Zebrafish embryos exposed to control (no added Cu) or 500 µg L^{-1} Cu at specific stages of embryonic development [hours post fertilization (hpf)]. (A) Cumulative mortality at 10 and 24 hpf (note, no embryos died between 24 and 72 hpf); (B). Heart rate in nine embryos from each beaker at 24 and 36 hpf; (C) percent of hatched embryos, determined from the proportion of embryos that survived to 72 hpf (mean numbers of surviving embryos that hatch: control, 18/21; 1-10 hpf, 8/12; 10-72 hpf, 12/14; 1-72 hpf, 1/4). Data are means ± S.E.M., (*n* = 3). The control and each treatment were conducted in triplicated test vessels, with each beaker consisting of 30 embryos. Different letters or numbers indicate significant differences between treatments were assessed by one-way ANOVA ($p < 0.05$). * Indicate significant differences between mortality at 10 and 24 hpf, or heart rate between 24 and 36 hpf within Cu treatment.

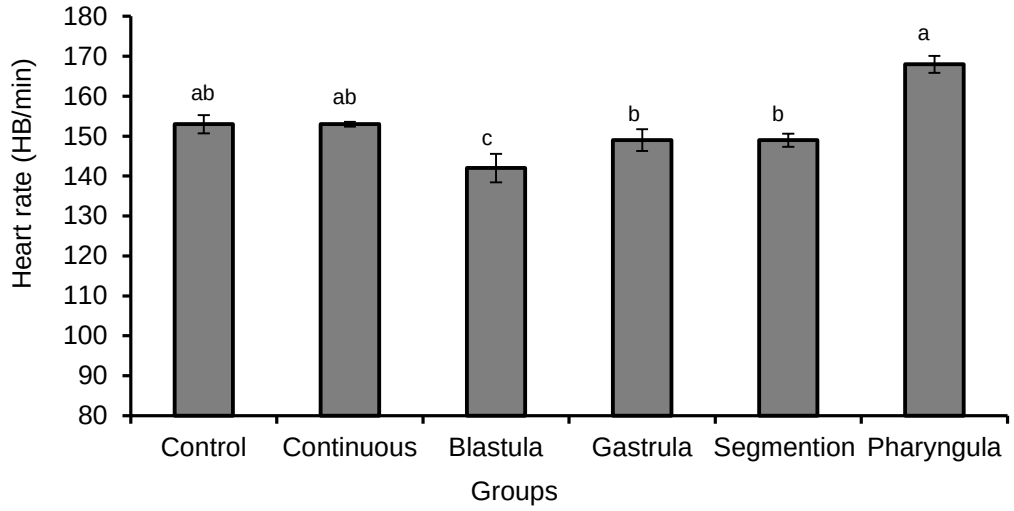

Figure 3.4 Heart rate (beats per minute) for zebrafish embryos exposed to either control (no added Cu) or 500 µg L^{-1} Cu^{2+} at specific stages of embryonic development [hours postfertilization (hpf)]. Exposure occurred in glass beakers (30 embryos per beaker), and the developmental stages at which embryos were exposed to 500 µg L^{-1} Cu were: blastula (2.25 - 5.25 hpf), gastrula (5.25 - 10.33 hpf), segmentation (10.33 - 24 hpf), and pharyngula (24 - 48 hpf). Heart rate was determined at 36 hpf, each bar is the mean of 10 embryos ± SE, and different letters indicate a statistically significant difference (ANOVA, $p < 0.05$).

3.3.3. Protective effect of calcium against copper toxicity

Mortality of embryos in both Ca conditions was between 28 and 31% in all beakers and there was no effect of Ca or Cu exposure conditions on embryo mortality (Fig. 3.5). There were no clear indications of abnormal development or morphology of embryos at the end of the exposure period (16 hpf).

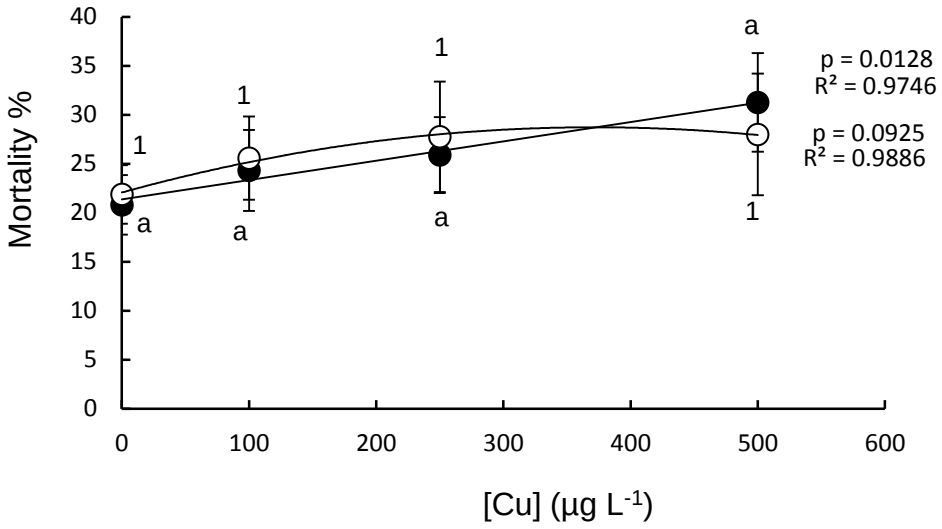

Figure 3.5 Cumulative mortality of zebrafish embryos exposed to copper sulphate in Plymouth freshwater at normal Ca concentration (black circles) or with 40 mg L^{-1} Ca added to the water (~ 60 mg L^{-1} Ca, open circles) at 16 hpf. Data are means ± S.E.M., *n* = 6 independent samples per treatment. The same letters or numbers within treatment indicate a non-significant (ANOVA, $p > 0.05$) effect of Cu exposure on the mortality. There was no effect on mortality of adding Ca at any Cu concentration (ANOVA, $p > 0.05$).

The concentration of copper in the embryos increased with the copper exposure concentration, but the apparent Cu accumulation differed between live and dead embryos (Fig. 3.6A). The amount of copper found in live embryos did not increase between copper exposures concentrations of 250 and 500 µg L^{-1}, indicating some attempt at regulation. However, in the dead embryos the Cu concentrations in/on the embryos followed the increase in exposure concentration (Fig. 3.6A). The interaction term copper concentration x embryo (alive or dead) was statistically significant (ANOVA, $p < 0.05$). Calcium concentrations in the embryos generally increased with external Cu concentration in the dead embryos, but Ca did not increase in live embryos and the amount of Ca in live embryos was less than in dead embryos at the higher Cu exposure levels (Fig. 3.6B). Copper exposure did not affect the concentration of Na^+ or K^+ in the embryos, but the concentrations of these electrolytes were higher in live embryos compared to dead embryos (Fig. 3.6C and D).

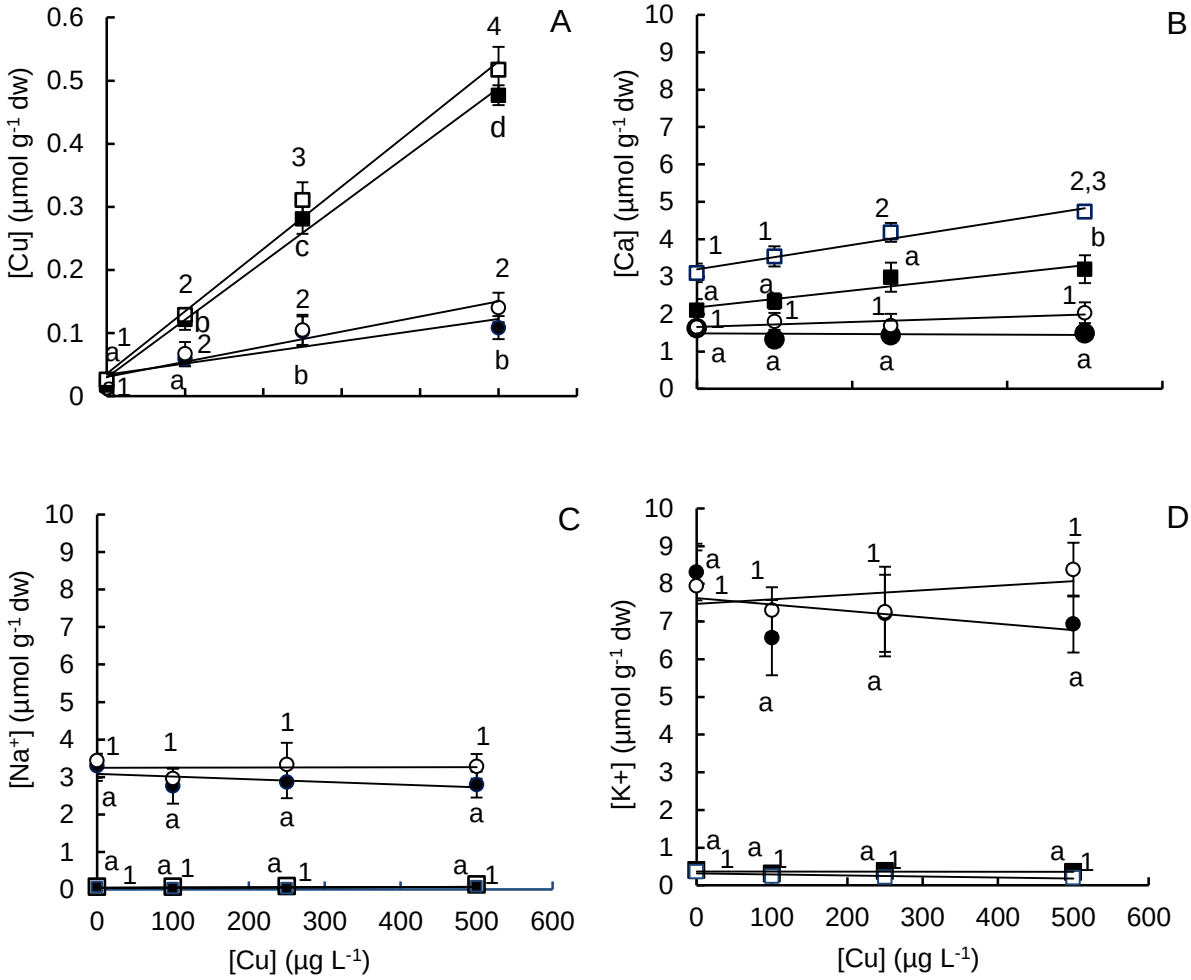

Figure 3.6 Metals concentrations [µmol metal g^{-1} dry weight of the embryo] in live (circles) and dead zebrafish embryos (squares) relative to Cu exposure concentration in Plymouth freshwater at normal Ca concentration (closed symbols) or with 40 mg L^{-1} Ca added to the water (~ 60 mg L^{-1} Ca, open symbols) at 16 hpf. The concentrations of copper (A), calcium (B), sodium (C), and potassium (D) in whole embryos are shown. The data points are means of 20 embryos ± S.E.M. Each exposure condition had six independent replicates and each replicate contained 170 embryos at the beginning of the exposure. Different letters (live embryos) or numbers (dead embryos) indicate a statistically significant effect (ANOVA, $p < 0.05$) of Cu concentration. * indicates a significant Ca addition-effect (2-way ANOVA, $p < 0.05$), which was only observed with respect to whole embryo Ca concentration.

3.3.4. Biochemical analysis

Exposure of embryos to dissolved copper in normal Plymouth water (without added calcium) significantly decreased the Na^+K^+-ATPase activity of the Cu-exposed embryos compared to the unexposed controls at the same Ca concentration (ANOVA, $P < 0.05$). However,, there was no statistically significant effect (ANOVA, $P > 0.05$) between different copper concentrations when the water Ca was normal (Fig. 3.7A). In the added calcium treatment, there was no effect of dissolved copper on Na^+K^+-ATPase activity, suggesting that the added Ca protected against Na^+ pump inhibition during Cu exposure (Fig. 3.7A). Total GSH was also measured in embryos during the same experiment. However, there was no statistically significant effect of copper exposure, or copper exposure with added calcium on total GSH; with values remaining at about 0.2-0.25 µmol g^{-1} $embryo^{-1}$ regardless of treatment (Fig. 3.7 B).

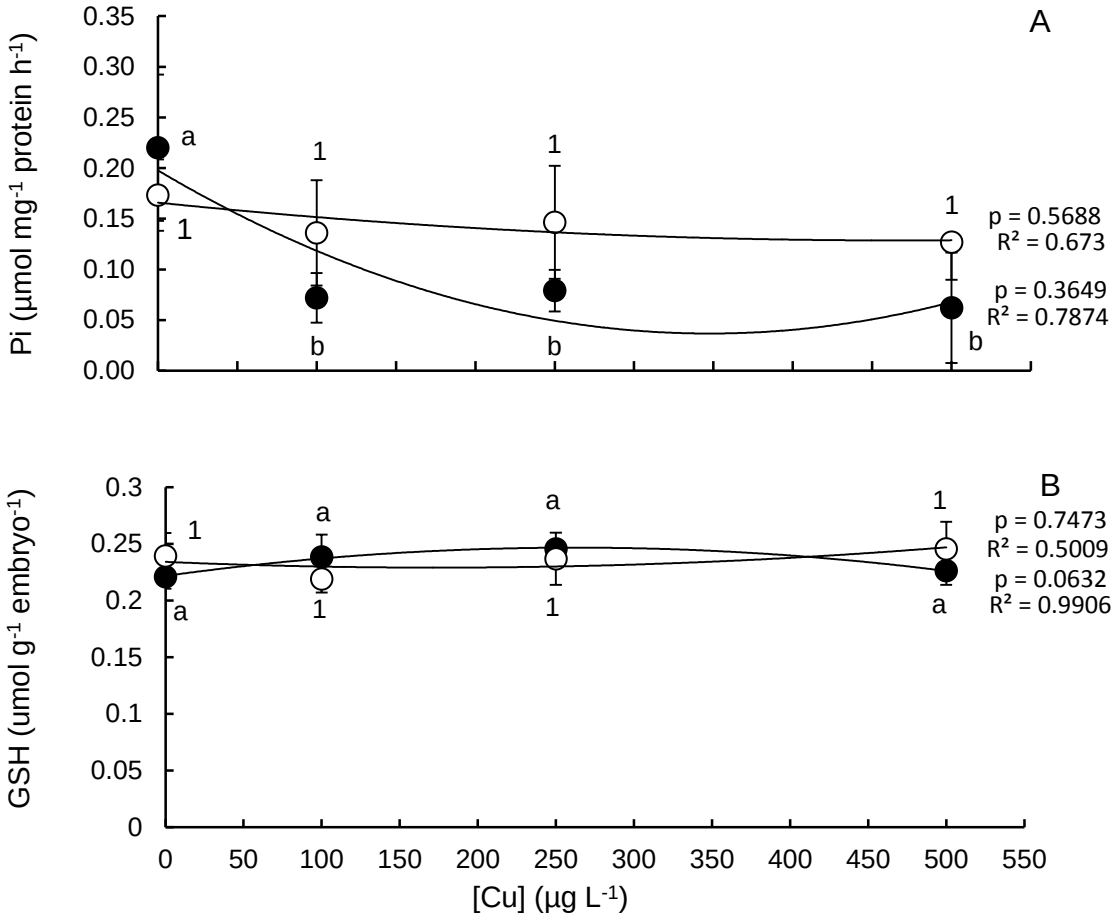

Figure 3.7 Activity of Na^+K^+-ATPase (A) and total glutathione (B), in zebrafish embryos after copper exposure with and without added calcium). Data are means ± SE (*n* = 6) with each replicate consisting of 170 embryos. Embryos exposed to copper without added Ca^{2+} (solid circles) and with added calcium (open circles) sampled at 16 hpf Different letters indicate statistically significant effect (ANOVA, $p < 0.05$) of Cu exposure without added Ca^{2+}, whereas same letters and numbers indicate non-significant effect of Cu with and without added calcium (ANOVA, $p > 0.05$).

3.3.5. Embryo length and yolk sac volume

Embryos exposed to Cu without added Ca^{2+} showed significant increase in the length of embryo (ANOVA, $p < 0.05$), whereas, added calcium did not show any significant effect (ANOVA, $p > 0.05$) on the length of embryos in all groups of treatment except those embryos exposed to 500 µg L^{-1} with added calcium (Fig. 3.8 A). The volume of yolk sac increased significantly (ANOVA, $p < 0.050$) in embryos exposed to Cu with and without added calcium, and was more pronounced in embryos exposed to Cu without added calcium (Fig. 3.8 B).

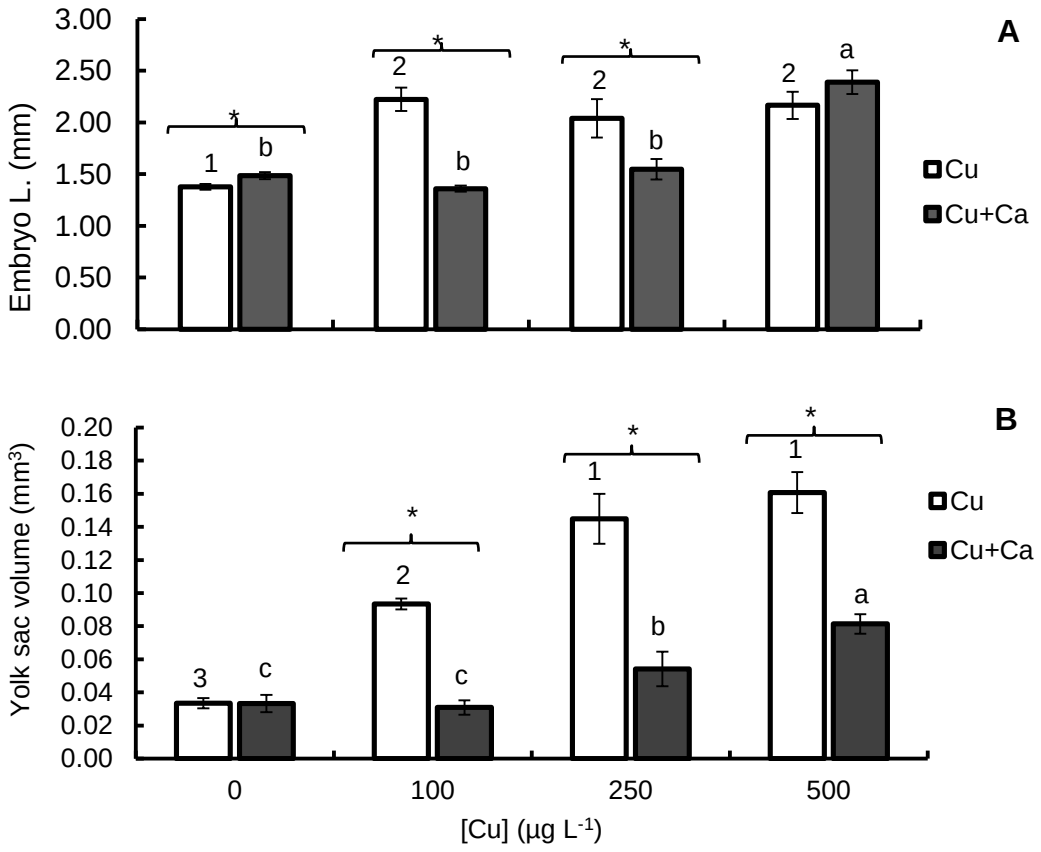

Figure 3.8 Length of embryo (A), and yolk sac volume (B), of zebrafish embryos followed copper exposure with and without added calcium. Data are means ± SE (*n* = 5). Embryos exposed to copper without added Ca^{2+} (white bar) and with added calcium (black bar). Different letters and numbers indicate significant differences within the groups (ANOVA, $p < 0.05$). * Indicate significant differences (ANOVA, $p < 0.05$) between embryos exposed to Cu with and without added calcium.

3.3.6. Gene expression

Some targeted gene expression was also conducted in the experiment that explored Ca modulation of Cu toxicity. The expression of *mt2* increased with the measured Cu concentration in the embryo when the Cu exposure was conducted at the normal Plymouth water Ca concentration. However, the addition of extra Ca abolished this effect, and with the higher Ca concentration in the water, the *mt2* expression of the embryos did not change (Fig. 3.9A). Conversely for transcripts of the *nkx2.5* gene, the expression increased with copper concentration in the presence of added calcium; but there was no effect on *nkx2.5* expression in embryos exposed to copper at the normal Plymouth water Ca concentration (Fig. 3.9B).

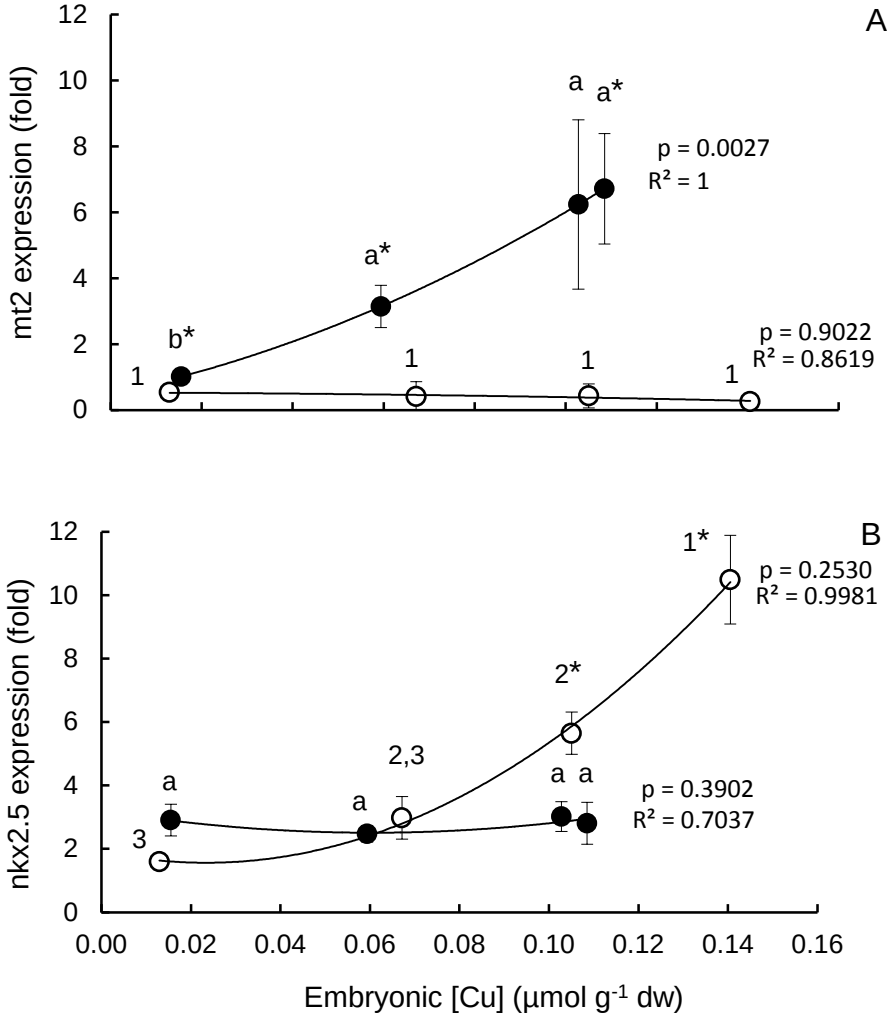

Figure 3.9 Expression of *mt2* (A) and *nkx2.5* (B) genes linking to Cu accumulation in live zebrafish embryos exposed to copper. Embryos were sampled at 16 hpf. Fold changes in gene expression were calculated by the $2^{\Delta\Delta Ct}$ method with ***β-actin*** used as a housekeeping gene. Data are means ± S.E.M., n = 3 samples per treatment. Embryos exposed to copper without added Ca^{2+} (solid circles) and with added calcium (open circles). Different letters and numbers indicate a significant effect of Cu with and without added Ca^{2+} from control (ANOVA, $P < 0.05$), whereas the same letters and numbers indicate a non-significant effect one way ANOVA ($p > 0.05$) within the treatments.

Transcripts of *nkx2.5* gene increased with copper concentration in the calcium added exposure and there was no effect on *nkx2.5* expression in embryos exposed to copper without added calcium (Fig. 3.9B).

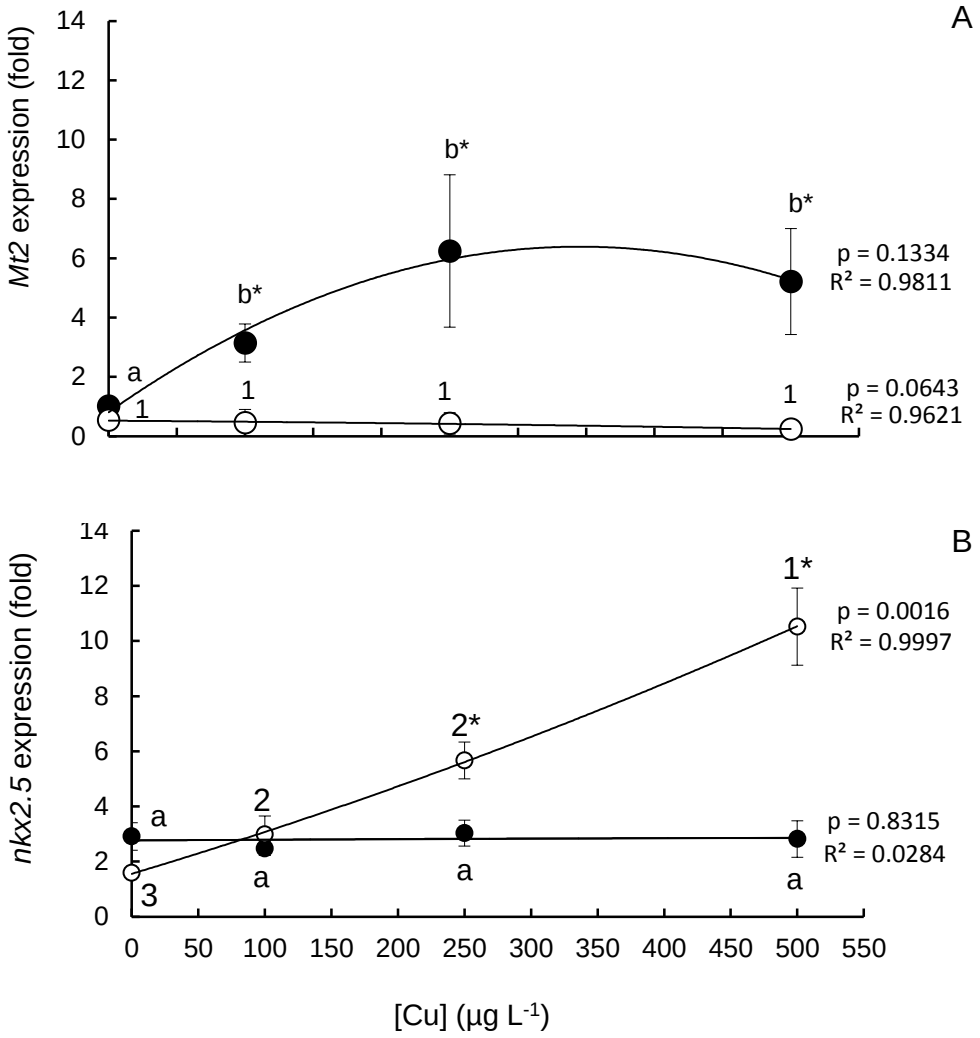

Figure 3.10 Expression of *mt2* (A) and *nkx2.5* (B) genes in embryos exposed to Cu with and without added Ca^{2+}. Embryos were sampled at 16 hpf. Fold changes in gene expression were calculated by the $2^{-\Delta\Delta Ct}$ method with β-actin used as a housekeeping gene. Data are means ± S.E.M., n = 3 samples per treatment. Embryos exposed to copper without added Ca^{2+} (solid circles) and with added calcium (open circles). Different letters indicate significant changes (ANOVA, $P < 0.05$). Different letters and numbers indicate a significant effect of Cu with and without added Ca^{2+} from control (ANOVA, $P < 0.05$), whereas the same letters and numbers indicate a non-significant effect (ANOVA, $p > 0.05$) within the treatments. * Indicates significant difference the expression of *nkx2.5* and *mt2* genes between embryos exposed to Cu with and without added Ca.

3.4. Discussion

3.4.1. Effect of copper on the early life stage

Zebrafish embryos showed an increase in mortality with respect to Cu concentration (0-1000 µg/L) and exposure time (10 h compared to 24 hpf, Fig. 1), in keeping with the notion of a dose-response relationship during acute toxicity. The estimate 24 h LC_{50} of 300 µg L^{-1} for zebrafish (Fig. 3.2) is within the range expected for fish embryos in fresh water. However, the most Cu-sensitive stage of embryonic development is less clear for zebrafish. In the present study, exposure to Cu throughout development (1-72 hpf) caused more mortality than exposure at 1-10 hpf or 10-72 hpf (Fig. 3.3A), and is consistent with the idea that a transient exposure to only part of the life stage is less hazardous than exposure throughout. Zebrafish exposed in the 1-10 hpf stage showed greater mortality than exposure in the latter half of development, suggesting the early embryo maybe more sensitive to Cu. The first 10 hpf (blastula and gastrula stages) is the time when hardening of the chorion occurs and has been noted previously as a period when fish embryos are vulnerable to various stressors (Weis & Weis, 1991). The hardness of the chorion plays a major role to protect the embryo by reducing the transport of toxicants including dissolved metals (Herrmann, 1993). This is confirmed in the present study, at 24 h there is no difference in the new mortality of fish that had been exposed at the 1-10 hpf stage compared to 10-72 hpf (Fig. 3.3A).

The cause of mortality during dissolved Cu exposure in the embryos of freshwater fish could be explained in terms of altered permeability of the vitelline membrane to toxic metals, leading to the disturbances in the exchange of cations between perivitelline fluid d and the water (Stouthart et al., 1996). The consequent swelling of the chorion with disturbance of the ionoregulatory system and electrolyte levels is probable causes of embryo mortality, as in adult fish (Handy et al., 2002). This suggestion of ionoregulatory toxicity was confirmed in the Ca addition experiment (see below).

Embryos exposed to Cu continuously had significant delayed hatching when compared to embryos of other groups. Delayed hatching has been observed previously in fish embryos exposed to Cu (Jezierska et al., 2009) and reduced hatching success has also been reported in various species including zebrafish (Johnson et al., 2007), rainbow trout *Oncorhynchus mykiss* embryos (Stasiūnaitė, 2005), and in red sea bream (Cao et al., 2010a). The hatching mechanism is a combination of enzymatic and mechanical effects and is initiated by secretion of chorionase from the hatching glands to disintegrate the zona interna of chorion during hatching (Yamamoto & Yamagami, 1975; Kapur & Yadav, 1982). Retardation of embryonic development and growth of the embryo by metals exposure has been suggested as reason for the observation of delayed hatching (Kapur & Yadav, 1982; Stasiūnaitė, 2005). Slowed development of the embryo will also delay the time at which movements of the embryo occur, and movements (e.g., twisting) of the embryo are required to tear open the zona externa of the chorion (Schoots et al., 1982).

The decrease in heart rate at 24 hpf was more pronounced in embryos exposed during 1-10 hpf compared to other groups, but this reduced heart rate was transient and heart rate did not differ at 36 hpf. Embryos exposed continuously to copper for 72 h showed increase in heart rate (Fig. 3.3B). Previous studies that report effects of copper exposure on heart rate have had mixed results, that appear to vary by Cu concentration and species of fish. At lower Cu concentrations, exposure to embryos of freshwater fish tend to show declining heart rate. For example, exposure of common carp embryos (*Cyprinus carpio*) to 50 µg L^{-1} Cu decreased the heart rate (Stouthart et al., 1996), and similar reduction in heart rate was observed in rainbow trout embryos exposed to 90 µg Cu L^{-1} (Stasiūnaitė, 2005). In red sea bream exposure of embryos to ≥ 80 µg L^{-1} and ≥ 60 µg L^{-1} Cu did not affect heart rate in embryos. This difference might be attributed to competition between the much higher Na^{+} concentrations in seawater with Cu^{2+} ion binding (Handy et al., 2003) in the polyanionic matrix of the perivitelline fluid. Nonetheless, once hatched, the red sea bream did show reduced heart rate in the newly hatched larvae (Cao et al., 2010a). In contrast, Johnson et al. (2007) observed increased stimulation of cardiac function

accompanied with increased heart rate in zebrafish embryos during exposure to 93, 327 and 464 µg Cu L^{-1}. The effect of such higher Cu exposure concentrations to the heart rate of freshwater adapted-embryos requires further investigation, but may relate to Na^+ pump inhibition and a subsequent Ca^{2+} influx on the Na^+/Ca^{2+} exchanger that leads to increased depolarisation of the sarcolemma (Ödblom and Handy, 2001). The aetiology of the gene expression of the cardiac ion transporters has not been established in fish embryos, but progressive recruitment of Na^+/Ca^{2+} exchanger activity may be a factor in the observed Cu-dependent increase in heart rate during development (Fig. 3.4).

Yolk sac volume and length of embryos was increased as Cu exposure increased with and without added calcium (Fig. 3. 8 A and B). The passive entrance of Cu which is concomitant with water infusion leads to increase yolk sac volume as well as increasing the embryonic length, thereby reducing the utilisation and reabsorption of yolk sac. Copper, as with other metals impaired the rate of embryonic development by increasing the yolk sac volume and decreasing the embryonic length (McKim and Benoit, 1971; Peterson et al., 1983; Johnson et al., 2007).

3.4.2. Effect of added calcium on copper toxicity and electrolytes concentrations

Although adding extra Ca to the water did not significantly affect Cu-dependent mortality (Fig. 3.5), there was a difference in the accumulation of metals in embryos exposed to added calcium (Fig. 3.6). Dead embryos that were exposed to Cu (with or without extra water Ca) showed increased embryonic Cu concentrations and depletion of Na^+ and K^+ in comparison to live embryos. Differences in electrolyte concentrations in live and dead embryos exposed to copper (regardless of Ca additions) is likely due to effects of active ionoregulation in live embryos (i.e., homeostatic retention of Na^+ and K^+) compared to a lack of active ionic regulation (i.e., passive ion exchange) in dead embryos.

Increased embryonic Cu accumulation was observed in live and dead embryos, and it was more pronounced in dead embryos. The increase in embryonic Cu concentration, especially in dead embryos, is best explained by passive entry of Cu^{2+} ions down the electrochemical gradient from the ambient water. It appeared that the hardness of the chorion (when the embryo was alive) was not enough to prevent the passive influx of copper ions (linear Cu accumulation, Fig. 3.6A). In moribund animals losing the ability to osmoregulate, the absorption of water by the perivitelline space leads to the swelling of the embryo; permitting more influx of water and ions by solvent drag (Peterson & Martin-Robichaud, 1982; Jezierska et al., 2009).

An increase of calcium concentration was seen in dead embryos, and this was more pronounced in embryos exposed to copper with added calcium (Fig. 3.6B). As the external Ca concentration is elevated the passive entry of Ca into the embryo will increase, filling the anionic residues in the perivitelline fluid (PVF) by simple ion exchange for ions such as H^+, Na^+ and K^+ in the PVF; so the passive potential becomes less negative (Shephard, 1987). Once the potential is zero or positive, then passive Cu entry will go down, as expected for all cations at that point in the external media (Shephard, 1987). Thus the effect of added Ca on passive potentials across the chorion may protect against increasing free metal ion activity of Cu in the embryo. However, Cu ions will bind avidly to the –SH groups in the mucoproteins of the PVF, and such changes in Cu ion activity are unlikely to be detected as Ca-dependent net changes in the Cu content of the embryos (only limited changes observed (Fig. 3.6A). In contrast, the increases of in embryonic calcium concentrations observed here may help to meet the requirements of the embryo at the early stage of development (Creton, 2004).

3.4.3. Effect of copper on Na^+K^+-ATPase activity, glutathione and *mt2* expression

Sodium pump activity decreased in embryos exposed to Cu in Plymouth freshwater containing normal Ca concentrations (Fig. 3.7). The impairment of Na^+K^+-ATPase activity could be caused by Cu accumulation in the embryos. It is well-known that the inhibition of Na^+K^+-ATPase activity by Cu ions can be attributed to the interaction between Cu^{2+} or Cu^+ ions with anionic sulfhydryl groups causing a defect in the tertiary structure of the enzyme, or compete between Cu ions with Mg^{2+} on the Mg^{2+} binding site of the enzyme (Li et al., 1998). The inhibition depends also on external Ca: with extra Ca added to the water the ATPase suffers no inhibition (i.e., water Ca is protective of the Na^+ pump). Increasing water Cu concentration in the absence of added Ca to the water, also depleted more of the total Na^+ and K^+ in the dead embryo (Figs. 3.6C and D), perhaps also limiting the substrate availability for the Na^+K^+-ATPase activity. The Na pump can also be inhibited by oxidative damage to the structure of the protein subunits of the enzyme. However, Cu-dependent oxidative stress seems unlikely.

Despite, increased embryonic Cu concentrations (Fig. 3.6A) the induction of GSH was not observed in the present study (Fig. 3.7B). Transient inhibition of the Na^+ pump without appreciable changes in the glutathione pool or evidence of oxidative damage to the tissue has also been observed in adult fish (African catfish, Hoyle et al., 2007). Consistent with the lack of change in the total GSH in the embryo, was an observed increase in *mt2* expression with Cu exposure (Fig. 3.8A). Metallothioneins are well known for their role as metal chelators in fishes (Hogstrand et al., 1991).

Copper exposure has been shown to induce the expression of metallothionein genes during the gastrulation and segmentation stages of fish development as the amount of maternal metallothionein reduces in the embryos (Riggio et al., 2003). The decrease in *mt2* expression with added calcium may be explained as result of the competition between effects of Ca and Cu ions for binding the

active site of metal response elements (MREs) in the genome of the zebrafish (Cheuk et al., 2008).

3.4.4. Effect of copper on *nkx2.5* expression

In the current experiment, embryos showed increase in *nkx2.5* expression (Fig. 3.1) relative to the time of embryonic development. The maximum expression was observed at in segmentation stage (10.33 - 24 h) at 16 hpf. The exposure of embryos to copper without added Ca to the water for up to 16 hpf showed no significant effect on *nkx2.5* gene expression, while a significant increase in *nkx2.5* gene expression appeared with added calcium; and coincided with increased embryonic copper accumulation. A tentative explanation is the increase in *nkx2.5* gene expression resulted from the competition between Cu and Ca during binding the Cys_2-His_2 zinc finger protein that switches on *nkx* genes (Lee et al., 1998), thereby, Ca^{2+}, as a second messenger, may play a role in gene expression (Hardingham & Bading, 1999). The addition of external Ca as well as the internal embryonic Ca may play a role to increase the expression of *nkx2.5* through the activation of RNA-binding proteins (Ikura et al., 2002). There are large numbers of RNA-binding proteins (RBPs) in the cells which activate the specific RNAs (Blech-Hermoni & Ladd, 2013). In contrast, *nkx2.5* expression may be increased to meet the requirement of the faster differentiation of myocardium myocyte in early embryonic development, as well as compensating for the probable decrease in gene expression which may accompany toxicity. Previous studies reported increased heart rates and cardiac arrhythmia concomitant with elevation of *nkx2.5* in zebrafish embryos exposed to hexabromocyclododecane (HBCD) for 72 h (Wu et al., 2013).

In conclusion, the mortality of zebrafish embryos increased with copper concentrations and embryos were most vulnerable to copper toxicity when exposure occurred during the gastrula and segmentation stages of development. Impairment of Na^+K^+-ATPase activity, and electrolyte imbalance together affect the survival of embryos as well as cardiac function. The addition of extra Ca to the water is protective of Cu toxicity in terms of restoring Na^+K^+-ATPase activity

and not requiring increased expression of the *mt2* gene and unchanged glutathione levels. The expression of *nkx 2.5* genes was not affected by Cu exposure alone, but becomes sensitive to Cu in the presence of added Ca. However, further work on the interaction of Cu and Ca on *nkx 2.5* gene expression in the heart is needed.

Chapter 4

The effect of waterborne silver on survival, ionoregulatory function, *mt2* and *nkx2.5* inductions in early life stage (ELS) of zebrafish *(Danio rerio)*

The effect of waterborne silver on survival, ionoregulatory function, *mt2* and *nkx2.5* inductions in early life stage (ELF) of the zebrafish (*Danio rerio)*

Abstract

The natural leaching of silver and anthropogenic activities may lead to the elevation of silver concentrations in the aquatic environment. Silver salts such as $AgNO_3$ are soluble and dissociate to yield free silver ions (Ag^+) which are known to cause ionoregulatory toxicity in adult fishes. However, the effects on fish embryos and their development are less well understood. The objectives of this study were to investigate the effects of dissolved silver on the survival and hatching success of zebrafish, and then to understand the sublethal responses that relate to defences against toxic metals (metallothionein expression), osmoregulatory disturbance (Na^+K^+-ATPase, electrolyte levels), oxidative stress (total glutathione), and cardiac development (*nkx2.5* gene). Embryos < 1 hpf were exposed to silver 0 (no added Ag), 2.5, 5, 7.5, 10 and 15 µg L^{-1} Ag as $AgNO_3$ for up to 72 h. Although, survival was not affected by increasing concentrations of total silver, a decrease in hatching and increase in heart beat was observed. Live and dead embryos were collected at 24 and 72 hpf for silver and electrolyte concentration determination, as well as biochemistry. A significant increase in embryonic silver accumulation in both live embryos aged 24 h and 72 h and dead embryos aged 24 h. The accumulation is more significant in 24 h live embryos than the other. Dead embryos and live embryos at 72 h exposed to Ag had lower Na^+ and K^+ concentrations than.24 h live embryos. Live embryos also showed a transient increase in Ca^{2+} concentration at 24 h in comparison to 72 h. There were no significant effect of Ag concentrations on Na^+K^+-ATPase activity, *Mt2*, total glutathione concentration in 24 and 72 h aged embryos, but a 4 fold increase respectively were seen in unexposed 72 h old embryos in comparison with unexposed 24 h old embryos. In contrast, *nkx2.5* gene expression significantly decreased 1 fold at 24 h old in embryos exposed to silver compared to controls. While, *nkx2.5* decrease 2 fold in 72 h unexposed embryos in comparison to 24 h unexposed embryos. Silver

toxicity is consistent with ionoregulatory toxicity in embryos and the cardiac gene involved in development is also affected.

4.1. Introduction

Silver is present naturally in the environment and some anthropogenic activities including mining and photoprocessing can increase concentrations of silver in surface waters (Wood et al., 1999). Recently, silver nanoparticles (Ag-NPs) have become used in various consumer products as antibacterial and antifungal agents; and these products can lead to the release of free silver ions (Ag^+) (Chen and Schluesener, 2008; Damm et al., 2008). The ionic form of silver (Ag^+) is bioavailable and extremely toxic to aquatic organisms (Hogstrand and Wood, 1998; Gorsuch et al., 2003); but Ag^+ complexes with chloride ions are considerably less toxic. Therefore, the toxicity of silver is a greater concern for freshwater organisms than for organisms that live at higher salt concentrations (e.g. estuarine and seawater environments), or waters that contain elevated levels of other Ag^+ complexing agents such as sulphides or dissolved organic matter (DOM) (Hogstrand and Wood, 1998) which bind to Ag^+ and reduce their availability (Wood et al., 1999).

Early life history stages of fish show greater sensitivity to silver than older fish (Nebeker et al., 1983). High mortality and delayed/decreased hatching are often reported although the severity of these effects varies according to fish species and silver concentration. Rainbow trout embryos showed higher mortality (56%) at day 32 post fertilization during chronic silver exposure (Guadagnolo et al., 2001), whereas, in acute exposure 100% mortality was reached between 8 – 17 days post fertilization (dpf) (Guadagnolo et al., 2000). Delayed hatching and reduced embryonic survival were related to aqueous silver concentration as observed by Powers et al. (2010) in zebrafish embryos exposed to 1 µmol Ag for 5 dpf.

The heart is the first organ to develop and function in vertebrate embryos during the embryonic development (Glickman and Yelon, 2002; Targoff et al., 2008), and is a target organ of metal toxicity (Cao et al., 2009; Li et al., 2009; Barjhoux et al., 2012). *Nkx2.5* is one of the essential genes to cardiac development and appears to be involved in initiating differentiation of cardiac cells at early stage of zebrafish embryonic of development (Chen and Fishman, 1996). It is well known that metals in adult aquatic organisms inhibit the activity of Na^+K^+-ATPase and disturb osmoregulatory functions and electrolyte levels; leading to cardiovascular disturbances and death of aquatic organisms (Hogstrand et al., 1996a; Webb and Wood, 1998; Vijayavel et al., 2007). Moreover, the Na^+ pump appears necessary for cardiac laterality and cardiomyocyte differentiation during the early stage of embryonic development (Shu et al., 2003). Due to the lack of the information about the effect of metals particularly silver exposure on the zebrafish early life stage. The current study may be potentially fruitful to understand the effect of silver on the formation and development of the zebrafish heart.

The aims of the study were, first, to investigate the toxic effect of waterborne silver exposure on the survival, hatching and cardiac function in early life stage of zebrafish; second, evaluate whether the silver exposure during the early life stages is associated with impairment of the osmoregulatory system (Na^+K^+-ATPase and electrolyte balance); third, determine the effect of silver on *mt2* induction and *nkx2.5* expression as one of genes which is essential for the cardiac formation and development.

4.2. Materials and Methods

4.2.1. Experimental design

Three experiments were conducted to establish the effect of Ag^{+} (as $AgNO_3$) on the early life stages of zebrafish. The experiments had the same design with each Ag^{+} exposure (0, 2.5, 5, 7.5, 10 and 15 µg L^{-1}) replicated in triplicate beakers (400 mL glass beaker with 300 mL exposure water) and each beaker stocked with 170 embryos. In each experiment, exposure was ended at 72 hpf (exposure was from 2-72 h post fertilization). All glassware was washed in 3% HNO_3 prior to the experiment. The stock solution 1 g L^{-1} Ag (as $AgNO_3$) was prepared by dissolving 1.577 g $AgNO_3$ (Sigma-Aldrich) in 1 L of deionized water. Silver concentrations were prepared from the stock solution prior the experiments. Exposure water was renewed every 24 h. The measured Ag concentrations (mean ± S.E.M., *n* = 6 beaker) of the nominal Ag concentrations in exposure solutions at 1^{st} h, 24^{th} h and 72^{nd} h are showed in (Table 2.1).

Table 4.1 Measured silver concentrations µg L^{-1} at 1st, 24th and 72nd h of exposure

Nominal Ag µg L^{-1} / Time/h	0	2.5	5	7.5	10	15
1st h	0.03 ± 0.001[a]	2.43 ± 0.03[a]	4.71 ± 0.04[a]	7.06 ± 0.10[a]	9.07 ± 0.14[a]	14.24 ± 0.11[a]
24th h	0.07 ± 0.00[a]	1.34 ± 0.04[b]	2.67± 0.13[b]	3.97 ± 0.15[b]	5.20 ± 0.09[b]	8.50 ± 0.12[b]
72nd h	0.05 ± 0.02[a]	0.80 ± 0.02[c]	1.53 ± 0.02[c]	2.45 ± 0.31[c]	3.51 ± 0.09[c]	5.98 ± 0.78[c]

Data are means ± S.E.M. (*n* = 6).
Different superscript letters a, b, c indicate significant Ag concentrations between the exposure solutions (ANOVA, $p < 0.05$).

Twenty live, 20 dead embryos and 10 hatched embryos after 24 h and 72 h of exposure were collected, respectively in 1.5 mL Eppendorf tubes for the determination of trace metal analysis. Sixty live embryos and 25 hatched embryos after 24 and 72 h of exposure were collected, respectively for the assessment of total protein concentration, total glutathione, and Na^+K^+-ATPase activity. One hundred live embryos and 30 hatched live embryos after 24 and 72 h of exposure were collected, respectively for gene expression. Twenty live embryos after 24 h of exposure were collected for the proteomics analysis. All the embryos sample were stored at -80 °C until they were used for analyses.

4.2.2. Embryonic electrolytes (Ca^{2+}, K^+, Na^+) and Ag analysis

The determination of trace metals in live and dead zebrafish embryos was performed according to Handy and Depledge (1999). Twenty live embryos (ww 35.4 ± 0.002 mg) and 20 dead embryos (ww 30.7 ± 0.004 mg) were collected in 1.5 ml Eppendorf tubes rinsed in 0.2 mmol L^{-1} EDTA solution, then processed for ion concentrations. Samples were dried in an oven (Gallenkamp Oven BS Model OV- 160) at 70 °C for 24 h. The dried weight of live and dead embryos

was (35.02 ± 0.002 mg) and (29.8 ± 0.004 mg), respectively. Whereas, the average of wet and dry weight of 10 live hatched embryos was 32.0 ± 0.001 mg and 30.7 ± 0.001 mg, respectively. The acid digestion of embryos was performed as in general method

4.2.3. Biochemical analysis and gene expression

For the biochemical analysis and gene expression, embryo samples were collected according to the design of the experiment, and stored at -80 °C in order to assay protein, Na^+K^+-ATPase and total glutathione. Biochemical analyses and gene expression were performed exactly as described in Chapter 2.

4.2.4. Gene expression

Preparation of embryonic homogenate and extraction of RNA were performed as described in chapter 2.

4.2.4. Statistical analysis

StatGraphic Plus version 5.1 was used to analyse all the data. One way analysis of variance was used to identify the effect of silver on embryos at 24 and 72 h. Fisher's least significant difference (LSD) test was used to identify differences between the means of treatments. Bartlett's test was used to check the validity of each ANOVA. To check the differences between 24 and 72 h live embryos and between 24 h live and dead embryos, two ways ANOVA was used. Results are presented as means ± S.E.M. For the calculation of fold-changes in the *nkx2.5* and *mt2* with normalization to βactin a comparative quantification ($2^{-\Delta\Delta Ct}$) was used (Henry et al., 2009).

4.3. Results

There were no changes in the temperature, dissolved oxygen (D.O.) or pH of exposure solutions at 1st, 24th, and 72nd hpf.

4.3.1. Effect of silver on embryo survival

There was no statistical significant effect (ANOVA, $p > 0.05$, Fig. 4.1A) of 24 h of silver exposure on mortality of zebrafish embryos in comparison to the unexposed controls. At the end of the experiment (72 hpf) the hatching success of all the treatment groups were determined (Fig. 4.1B). At 72 hpf the embryos subject to Ag exposure (1 – 72 hpf) showed lower hatching rate concomitant with increased Ag concentrations. Embryos subject to 10 and 15 µg L^{-1} Ag had lower hatching rate in comparison to all other groups (ANOVA, $p < 0.05$, Fig. 4.1 B). Heart rate was also assessed at 36 hpf when the heart is usually anatomically developed. Exposure of embryos to the 10 and 15 µg L^{-1} Ag resulted in a significant increase in heart rate (one way ANOVA, $p < 0.05$, Fig. 4. 1C) at 36 hpf in comparison to the unexposed control and all the other groups.

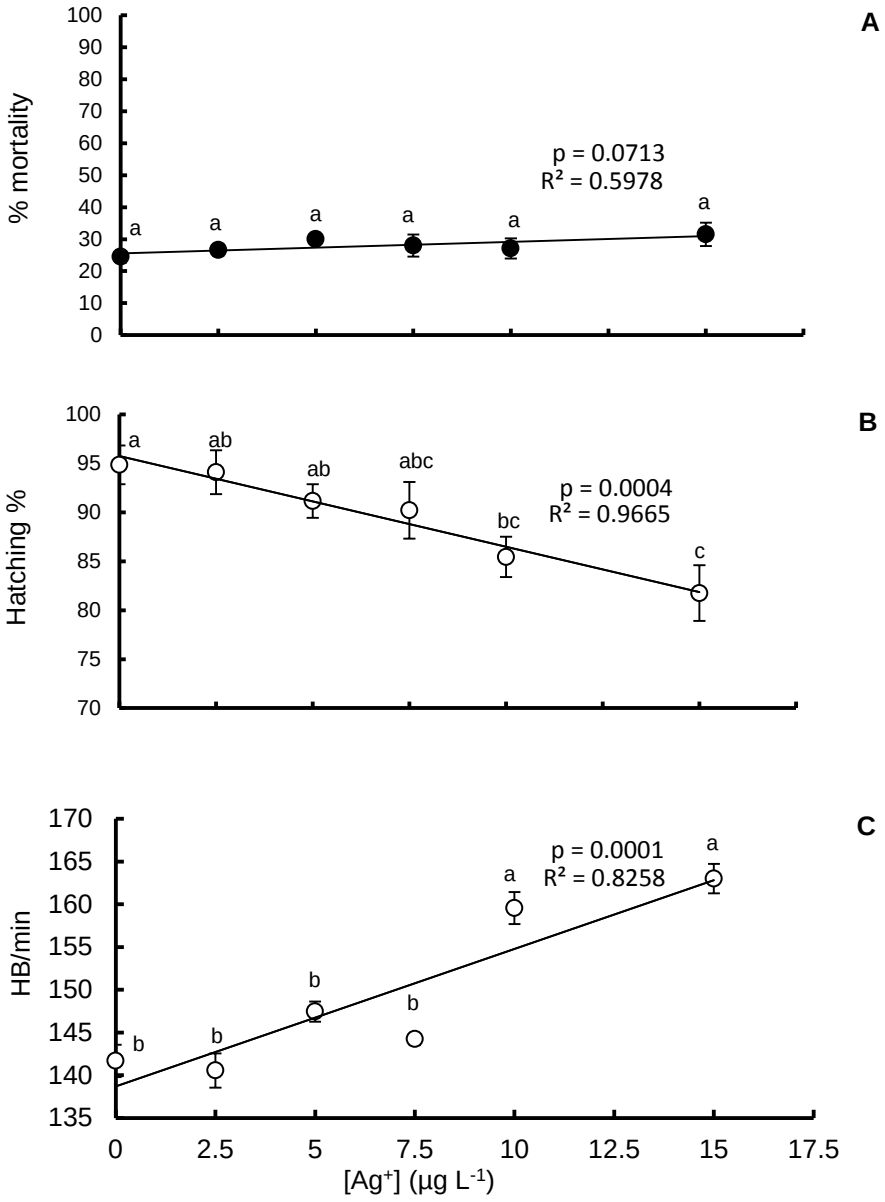

Figure 4.1 Embryos exposed to Ag^{+} as $AgNO_3$. The control and each treatment consisted of 170 embryos exposed in a glass beaker with six replicate beakers for each condition. Mean mortality (±SE) of embryos (A) was assessed in each beaker at 24 hpf. At 72 hpf, the number of embryos that hatched (B) was determined as a percentage of those embryos that survived to 72 hpf. Mean heart rate (±SE) of embryos (C) was assessed in nine embryos from each beaker at 36 hpf. Data are means ± S.E.M, *n* = 6 beakers per treatment. Different letters denote statistically differences between concentrations (ANOVA, $p < 0.05$). Similar letters denote statistically no significant effect (ANOVA, $p > 0.05$).

4.3.2. Embryonic electrolytes and Ag^+ concentrations

Embryonic metal concentrations were confirmed by ICP-MS. There were significant increases in embryonic silver concentrations in 24 h live and dead and 72 h aged live embryos as exposure silver concentrations increased. The increase was more pronounced in 24 h live embryos in comparison with 24 h dead embryos and 72 h live and (ANOVA, $p < 0.05$, Fig. 4.2 A, and Fig. 4.3 A), respectively. Decreases in embryonic Na^+, K^+ and Ca^{2+} concentrations were observed in 24h aged dead embryos and 72 h aged live embryos. The decreases were more pronounced in dead embryos (ANOVA, $p < 0.05$, Fig. 4. 2B, C, D, and Fig 4.3B, C, D), respectively. The decreases were more pronounced in 24 h aged dead embryos than 72 h live hatched embryos.

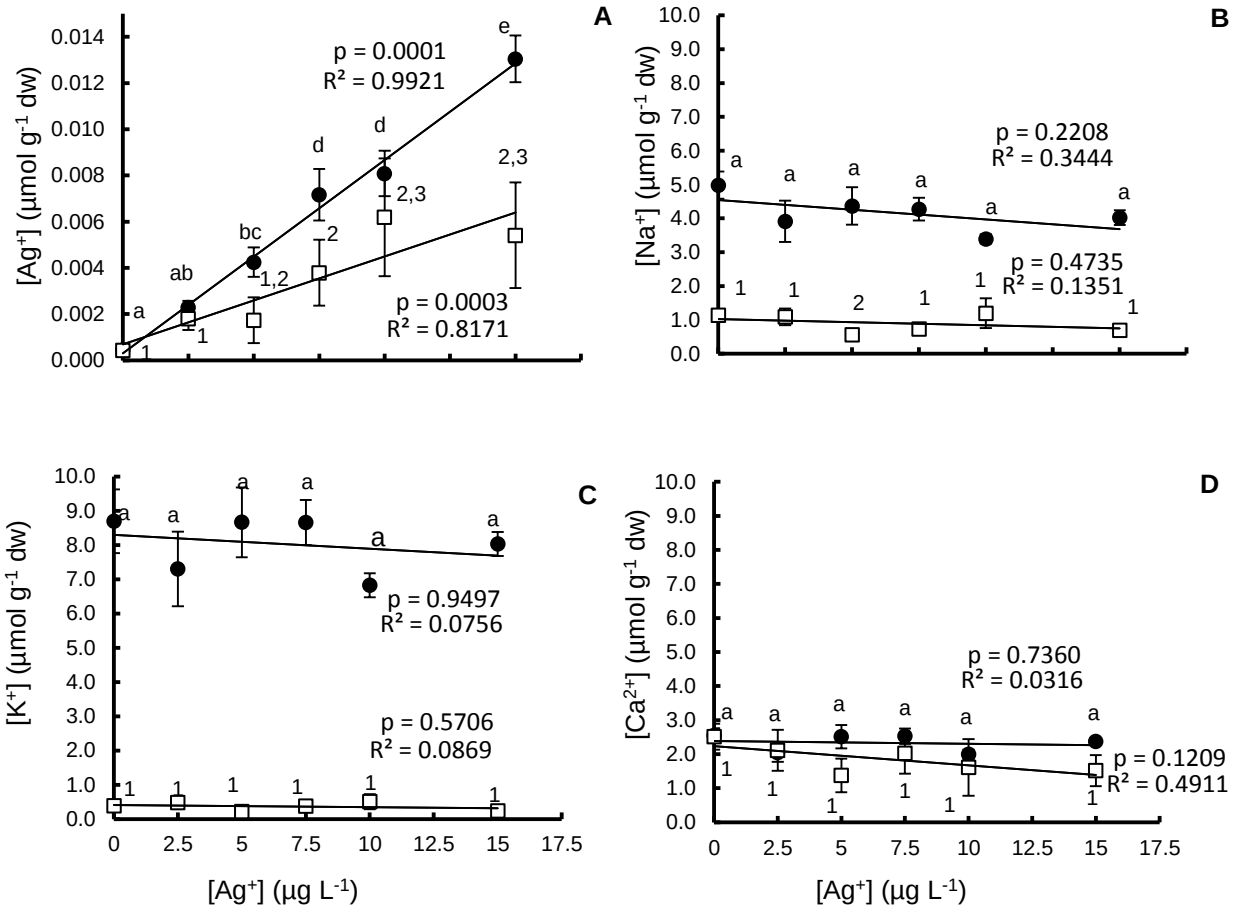

Figure 4.2 Metals concentrations [µmol metal g^{-1} dry weight of the embryo (±SE)] in live (solid circle), and dead (open square) zebrafish embryos at 24 hpf relative to the nominal total Ag exposure concentrations. The concentrations of measured silver (A), sodium (B), potassium (C), and calcium (D) were assessed in embryos exposed to $AgNO_3$. Each exposure condition had six independent replicates and each replicate contained 170 embryos at the beginning of the exposure. The number of live and dead embryos analysed for each condition was (20). Different letters and numbers denote statistically differences between concentrations (ANOVA, $p < 0.05$). Similar letters and numbers denote no statistically differences between concentrations (ANOVA, $p > 0.05$).

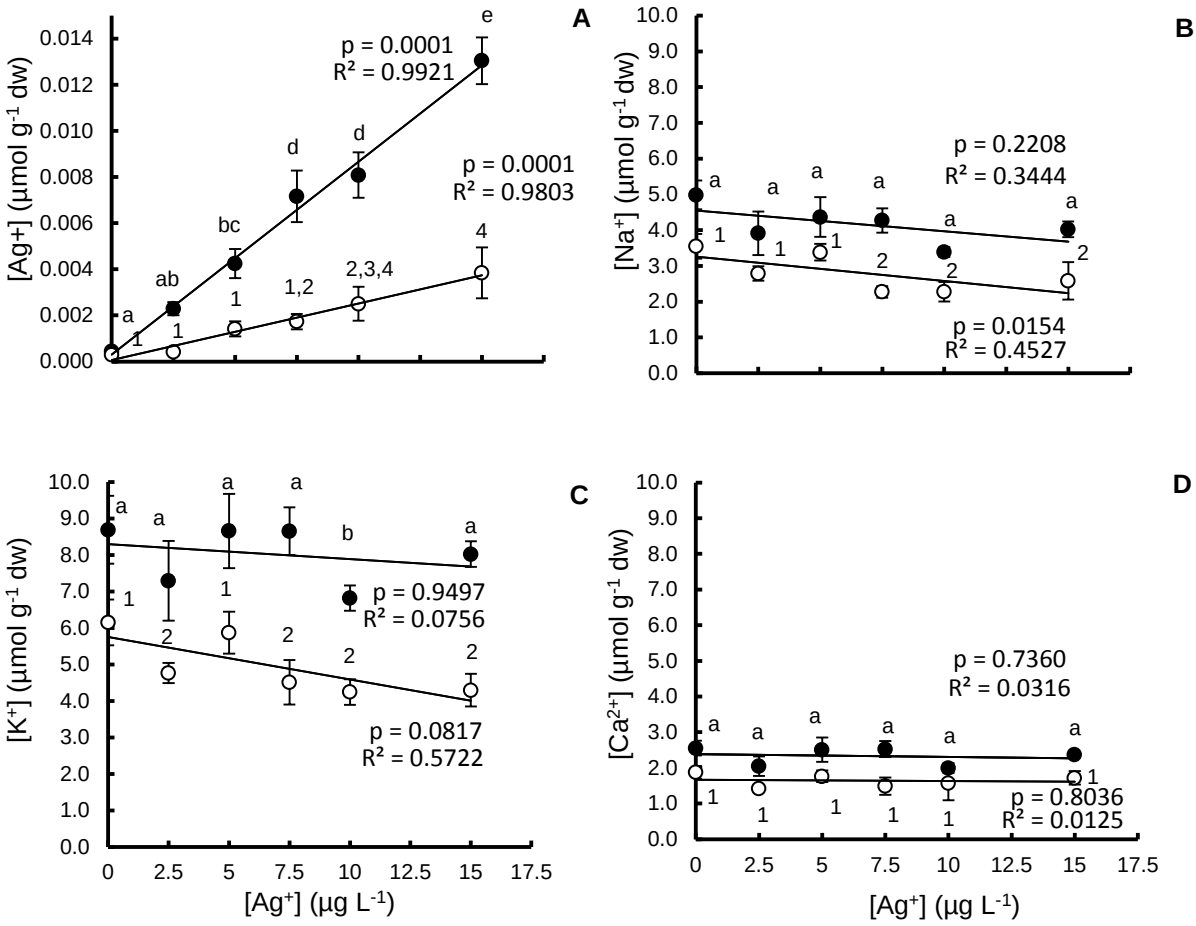

Figure 4.3 Metals concentrations [µmol metal g^{-1} dry weight of the embryo (±SE)] in live zebrafish embryos at 24 hpf (solid circle), and 72 hpf (open circle) relative to Ag exposure concentrations. The concentrations of silver (A), sodium (B), potassium (C), and calcium (D) were assessed in embryos exposed to $AgNO_3$. Each exposure condition had six independent replicates and each replicate contained 170 embryos at the beginning of the exposure. The number of live embryos analysed for each condition was (20). Different letters and numbers denote statistically differences between concentrations (ANOVA, $p < 0.05$). Similar letters and numbers denote no statistically differences between concentrations (ANOVA, $p > 0.05$).

4.3.3. Biochemical analysis

Although, there was increased in embryonic silver concentration in live embryos after 24 and 72 h of exposure (Fig. 4. 4. 2A), there was no statistically significant effect (ANOVA, $P > 0.05$) on Na^+K^+-ATPase activity in both 24^{th} and 72^{nd} h embryos (Fig. 4. 4A). Total GSH was also measured in embryos during the same experiment. However, there was no statistically significant effect (ANOVA, $P > 0.05$) of silver exposure on total GSH in both 24^{th} and 72 h embryos (Fig. 4. 4B). However, the increased embryonic silver concentrations had no significant effect on Na^+K^+-ATPase activity and total glutathione in both 24 h and 72 h hatched embryos (Fig. 4. 5 A and B). Statistically significant effect of silver exposure on Na^+K^+-ATPase activity and total glutathione was recorded in 72 h hatched embryos (two way ANOVA, $p < 0.05$, Fig. 4.4 A, B,) in comparison with the 24-h aged embryos.

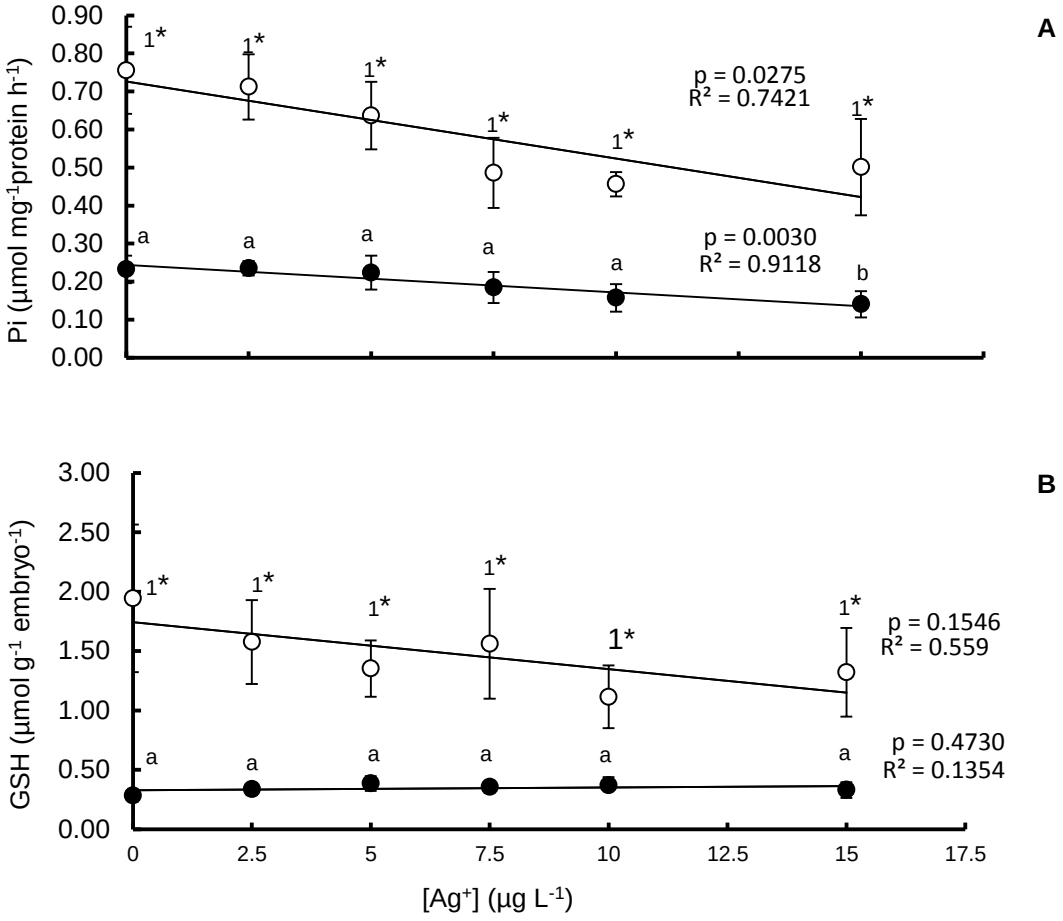

Figure 4.4 Mean Na^+K^+-ATPase activity (A), total glutathione (B), in live zebrafish embryos at 24 hpf (solid circle) and 72 hpf (open circle) following silver exposure. Data are means ± SE (*n* = 6) with each replicate consisting of 170 embryos. Although, the ANOVA test showed no statistical significance effects of Ag on Na^+K^+-ATPase activity and total GSH levels (ANOVA, $p > 0.05$). LSD showed significant effect of the 10 µg L^{-1} on Na^+K^+-ATPase in comparison to control. *Indicate the significant differences (ANOVA, $p < 0.05$) between 24 and 72 h aged embryos.

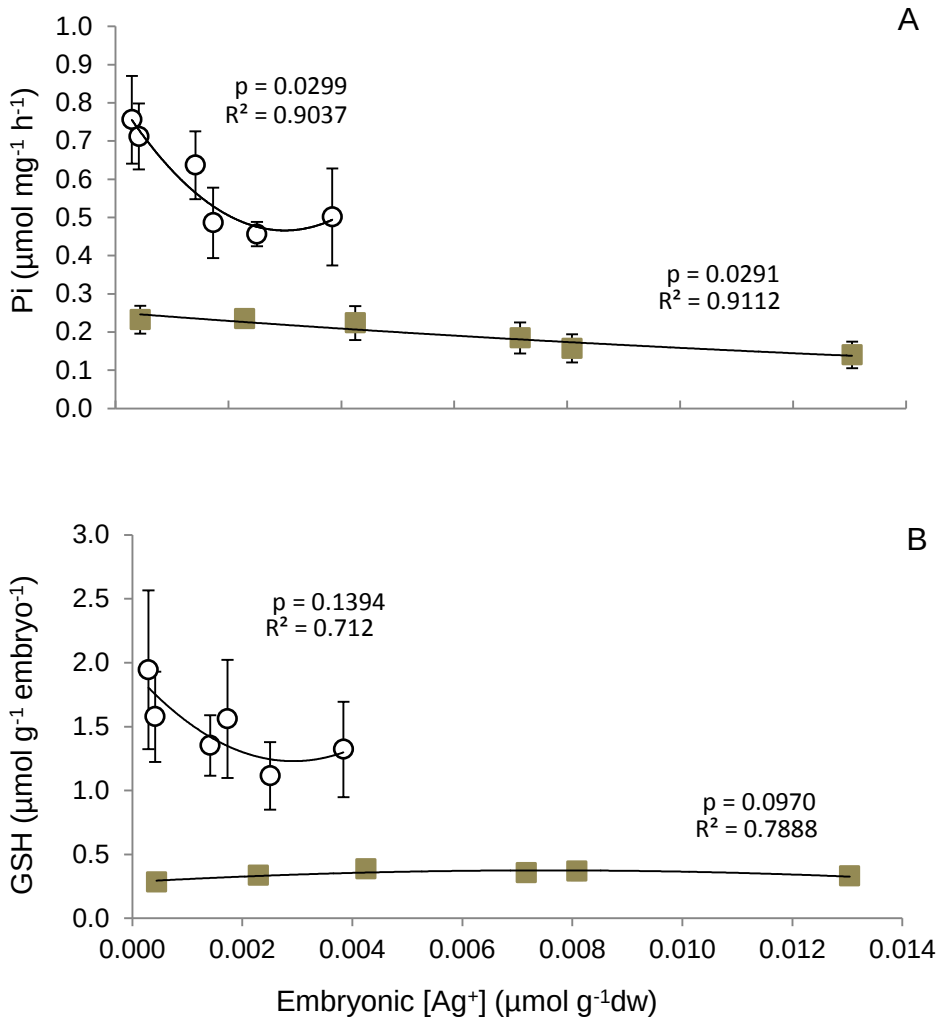

Figure 4.5 Mean Na^+K^+-ATPase activity (A), total glutathione (B), in live zebrafish embryos at 24 hpf (solid square) and 72 hpf (open circle) relative to embryonic Ag concentrations. Data are means ± S.E.M (*n* = 6) with each replicate consisting of 170 embryos.

4.3.4. Embryo length and yolk sac volume

The length of embryo and yolk sac volume in exposed embryos did not show significant differences (ANOVA, p > 0.05) (Figure 5 A, B).

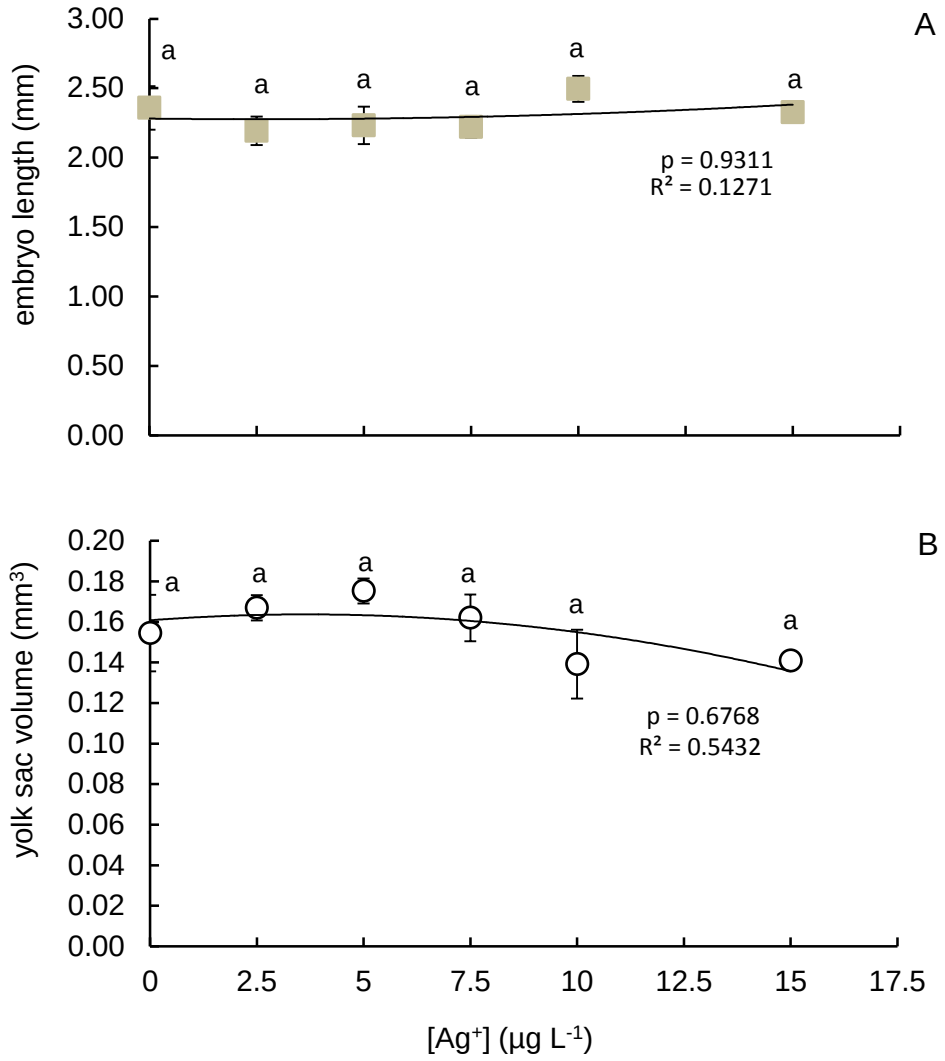

Figure 4.6 Length of embryo (mm) (A), and yolk sac volume (mm^3) (B), of zebrafish embryos followed silver exposure. Data are means ± S.M.E (*n* = 5). Similar letters indicate non-significant effect of silver (ANOVA, p > 0.05).

4.3.5.*Mt2* and *nkx2.5* expressions

The expression of *mt2* decreased with the increased silver concentrations in the 24 h embryo (ANOVA, $p < 0.05$, Fig. 4.7 A), whereas, the expression of *mt2* had no significant increased (ANOVA, $p > 0.05$, Fig.4.7 A) in 72 h hatched embryos with increased silver concentrations. In contrast, the expression of *nkx2.5* decreased significantly (ANOVA, p 0.05, Fig. 4.7 B) in 24 h embryos with increased silver concentrations, whereas no effect was seen in 72 h aged embryos. However, A significant increased expression of *mt2* (4 fold) and decreased expression of *nkx2.5* (3 fold) were observed in unexposed 72 h hatched embryos in comparison with unexposed 24 h embryos (two way ANOVA, $p < 0.05$, Fig. 4.7A and B).

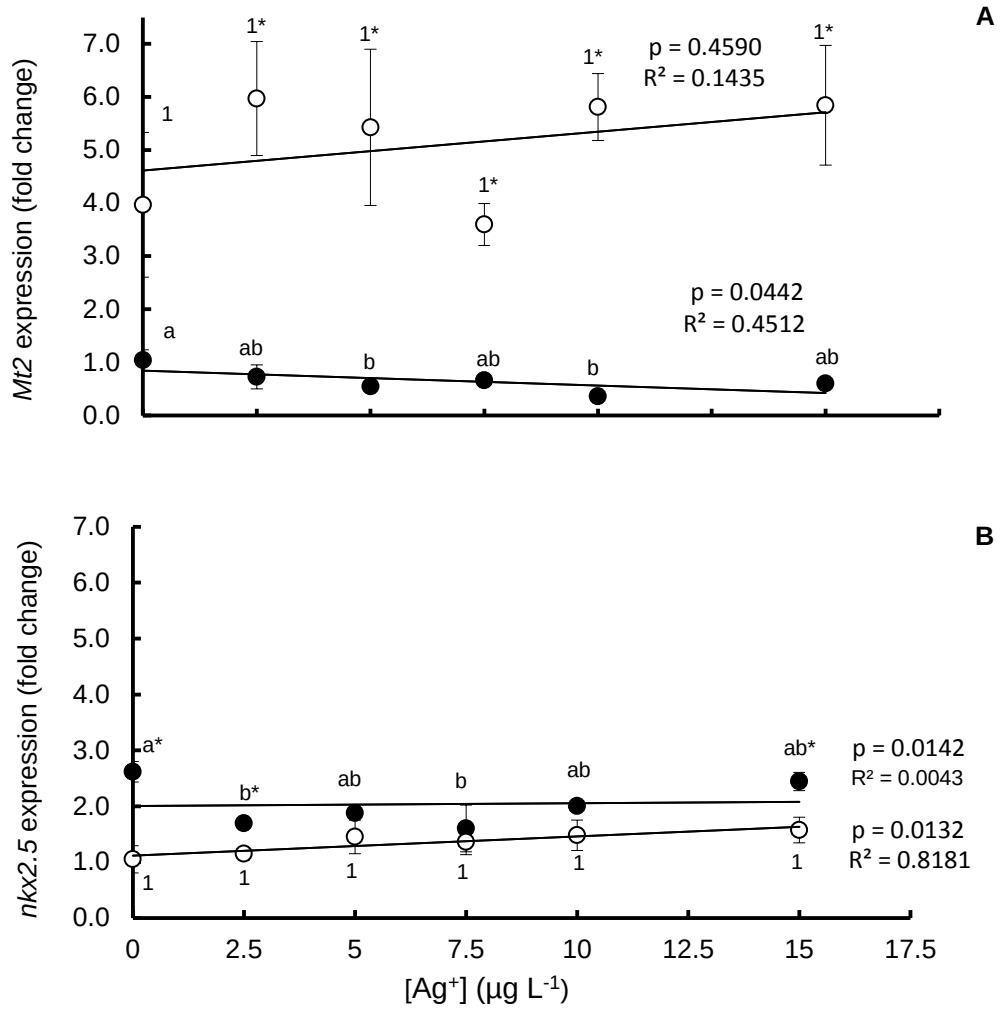

Figure 4.7 Expression of *mt2* (A) and *nkx2.5* (B) relative to silver concentrations. Embryos were sampled at 24 hpf (solid circle) and 72 hpf (open circle). Fold changes in gene expression were calculated by the $2^{-\Delta\Delta Ct}$ method with β-actin used as a housekeeping gene. Data are means ± S.E.M., *n* = 3 samples per treatment. Different letters indicate a significant effect within the concentrations (ANOVA, P < 0.05). Same numbers indicate no significant effect (ANOVA, p > 0.05) within the concentrations. * Indicate the differences in the expression of genes between 24 and 72 h aged embryos.

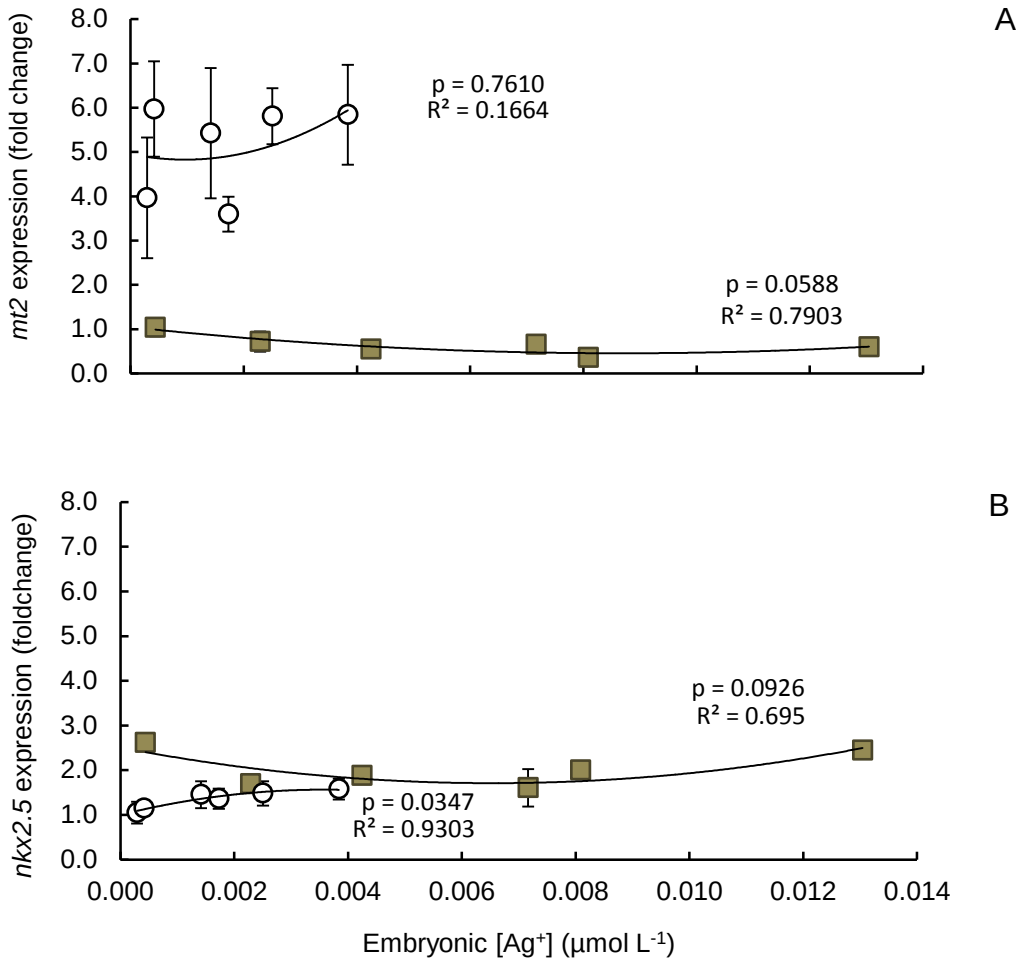

Figure 4.8 Expression of *mt2* (A) and *nkx2.5* (B) relative to the embryonic silver concentrations. Embryos were sampled at 24 hpf (solid square) and 72 hpf (open circle). Fold changes in gene expression were calculated by the $2^{-\Delta\Delta Ct}$ method with β-actin used as a housekeeping gene. Data are means ± S.E.M., *n* = 3 samples per treatment.

4.4. Discussion

4.4.1. Mortality and embryonic silver concentration

Exposure of embryos to silver concentrations for 24 h did not show any statistically significant effect on mortality, despite the dead embryos showing increases in silver concentration in comparison to the control. The early stage of embryonic development, particularly the blastula and gastrula stages are considered vulnerable to various stresses and represent the stages when hardening of the chorion occurs (Weis and Weis, 1991). Although, the hardness of the chorion plays a major role in the protection of the embryo from the absorption of toxicants, including dissolved metals (Herrmann, 1993), the consequent chorion swelling may be assist in the transfers of toxicants by altering the permeability of the vitelline membrane to toxic metals leading to the disturbances in the exchange of cations between perivitelline fluid and the water (Stouthart et al., 1996). The ionoregulatory disturbance and electrolytes imbalance are the probable cause of embryo mortality as in adult fish (Hogstrand and Wood, 1998; Guadagnolo et al., 2000). Acute exposure of rainbow trout embryos to 0.11, 1.55 up to 14.15 µg L^{-1} Ag for 5 days had no significant effect on mortality (Guadagnolo et al., 2000). Chronic exposure of rainbow trout embryos to measured Ag concentrations 0.117, 1.22, for 32 days had no effect on embryos mortality, whereas, the mortality reached 56% by day 32 post fertilization in embryos when exposed to 13.51 µg L^{-1} for the same period (Guadagnolo et al., 2001).

The increase Embryonic silver concentration was more pronounced in 24 h aged live embryos than 24 h dead and 72 h live hatched embryos (Fig. 4.2A and 4.3A). The increased indicates that the 24 h live embryos were able to regulate the accumulation of silver to more than 24 h dead and 72 h live embryos. Embryos may be dead due to simply passive ion binding processes or they took up more silver when alive. The accumulation of silver within the embryo may be as result of the absorption of water by perivitelline space leading to the swelling of the chorion, which permits more influx of water and

ions into the embryo during the first hours post fertilization (Peterson and Martin-Robichaud, 1982; Selderslaghs et al., 2009). However, the presence of −SH group as a constituent of chorion proteins able to bind metal cations such as silver, copper and mercury as Na^+ antagonists and assist the entrance of silver as well other ions into the embryo (Rombough, 1985; Brivio et al., 1991; Sugiyama et al., 1996).

Increased embryonic Ag concentrations which concomitant the increased Ag exposure did not showed significant effects on the length of embryo and yolk sac volume. Silver exposure may be not enough to affect the embryonic length and yolk sac volume, and thereby impair the development of embryo.

4.4.2. Effect of silver on hatching mechanism

Embryos exposed to 10 and 15 µg L^{-1} Ag for 72 h had more pronounce delay hatching in comparison to other groups. Delayed hatching has been observed previously in zebrafish embryos exposed to 1 µM Ag for 5 dpf (Powers et al., 2010). It is well known the hatching mechanism is a combination of enzymatic and mechanical effects and is initiated by secretion of chorionase from the hatching glands to disintegrate the zona interna of chorion during hatching (Kapur and Yadav, 1982; Yamamoto and Yamagami, 1975; Fraysse et al., 2006). The impairment and retardation in the growth, as well as the disturbances in the synthesis and secretion of chorionase from hatching glands to disintegrate the zona interna of chorion can lead to decrease and delay hatching (Yamamoto and Yamagami, 1975; Kapur and Yadav, 1982). Retardation of embryonic development and growth of the embryo by metals exposure has also been suggested as reason for the observation of delayed hatching (Kapur and Yadav, 1982; Stasiūnaitė, 2005). Slowed development of the embryo will also delay the time at which movements of the embryo occur, and movements (e.g., twisting) of the embryo are required to tear open the zona externa of the chorion (Schoots et al., 1982).

4.4.3. Effect of silver on heart rate

Embryos exposed to silver for 72 h showed increase in heart rate, which was pronounce in embryos exposed to 10 and 15 µg L^{-1} (Fig. 1C). The increased in heart rate might be as a response to the toxic effect of metals as in adult fish. Fish are able to cope with disturbances, including physical and chemical stressors, to maintain their homeostatic state (Barton, 2002). The increase in heart rate may be related to the inhibition of Na^+K^+-ATPase pump leading to alteration in the K^+: Na^+ ratio due to the leak of K^+ to the extracellular fluid (ECF) and increased intracellular Na^+. Such changes concomitant with a subsequent Ca^{2+} influx on the Na^+/Ca^{2+} exchanger lead to increased depolarisation of the sarcolemma (Ödblom and Handy, 2001).

4.4.4. Embryonic electrolyte concentrations

Decrease in Ca^{2+}, K^+, and Na^+, concentrations were seen in dead 24 h and 72 h live hatched embryos (Fig. 4.2 and 4.3, respectively). The decrease may have occurred as a result of osmoregulatory disturbances. due to the effect of Ag on ionoregulatory in live 72 h hatched embryo and passive ion exchange in 24 h dead embryos. However, electrolyte leak post mortem is normal and occurred as a passive loss by diffusion to the freshwater versus the passive influx of Ag by diffusion into the dead embryos. On the other hand, the decrease in Ca may be occurred due to the release of Ca from Ca^{2+} release channel (CRC) and Ca^{2+}-ATPase which are inhibited by the interaction of Ag with their –SH groups (Tupling and Green, 2002).

4.4.5. Effect of silver on Na^+K^+-ATPase, glutathione and *mt2*

Although, there was an increase in embryonic silver concentrations, no significant impairment of Na^+K^+-ATPase was seen in embryos exposed to silver for 24 h, and 72 h (Fig. 4.4 A). Proteomics analysis of embryonic homogenate of embryos exposed to 5 and 15 µg L^{-1} Ag for 24 h showed the induction of ATP

synthase subunit α and β with increased Ag concentrations that assist in synthesis or substitute the impaired Na^+K^+-ATPase molecules. In adult fish the impairment of the Na^+K^+-ATPase is a key mechanism of silver toxicity and that of other toxic metals (Morgan et al., 1997; Bury and Wood, 1999; Satyavathi and Prabhakara Rao, 2000; Bianchini and Wood, 2002).

The number, location, and well-developed ionocytes that are abundant with Na^+K^+-ATPase play an important role to control the osmoregulation. The presence of ionocytes (chloride cells) are exist on the yolk sac membrane and body skin tegument considered as a substitute of the undeveloped osmoregulatory organs such as gills and kidneys (Tytler et al., 1993; Kaneko et al., 2002; Varsamos et al., 2002; Sucré et al., 2010). Silver like other transition metal ions has affinity to bind the −SH group of α subunit of Na^+K^+-ATPase molecules. The competition between Ag^+ and Mg^{2+} on the binding sites on the α subunit of the Na^+K^+-ATPase molecule prevents the hydrolysis of ATP, which is as necessary step in the activation of Na^+K^+-ATPase (Ferguson et al., Hogstrand and Wood, 1998). The no significant impairment of Na^+K^+-ATPase activity during the development of 24 h embryos may be explained by low and under developed ionocytes, although there is a gradual maturation of ion transporting epithelial associated with Na pump activity (Tytler et al., 1993; Varsamos et al., 2005). In contrast, although there was no significant effect of $AgNO_3$ concentrations on Na^+K^+-ATPase activity in live 72 h hatched embryos, but there was increase in Na^+K^+-ATPase activity in comparison to live 24 h embryos (Fig. 4.4A). The presence of high number and well-developed ionocyte cells as a main site of the osmoregulation in 72 h hatched embryos may be explain such significant increase in Na^+K^+-ATPase activity.

Although, increased embryonic silver concentration (Fig. 4.2 A) at 24 h and 72 h embryos, there was no significant induction of total GSH levels (Fig. 4.4B). No significant effects on Na^+ pump and without appreciable changes in the metallothionein indicate there was no evidence of oxidative damage in the present study. On the other hand, increased induction of total glutathione levels in 72 h hatched compared to 24 h aged embryos probably due to the well-

development of the enzymatic system of the synthesis of the glutathione. However, the lack of change in the level of total glutathione may be due to the low concentrations of the exposed silver which is under thresholds to trigger the induction of glutathione and metallothionein (Atli and Canli, 2008).

Mt2 expression was showed decrease in 24 h embryos in comparison to control (Fig. 4.7A), although, there was increased in embryonic Ag accumulation (Fig. 4.2A). The expression may be interfere with presence of high maternal metallothionein that interact with accumulation of embryonic silver. Silver exposure for 72 h did not show significant induction of *mt2* expression although there was increased embryonic Ag accumulation and reduction in the amount of maternal metallothionein at this stage of embryonic development (Riggio et al., 2003). The nominal concentrations as well as the increased embryonic silver concentrations may be not enough to trigger the expression of *mt2*.

4.4.6. Effect of silver on *nkx2.5* expression

The exposure of embryos to silver for up to 24 was showed significant decrease on *nkx2.5* gene expression, while no significant effect appeared in embryo exposed to Ag up to 72 h (Fig. 4.7 B). It is well known that silver ions possess affinity to bind -SH group in many enzymes leading to the interference with protein function (Lee and Schedl, 2006; Glisovic et al., 2008). The increased embryonic silver concentrations may play a role as an inhibitor to the of RNA-binding proteins activity. The latter are considered to have a key role in RNA transcription. In contrast, cysteine, histidine (C_2H_2) zinc finger protein play an important role as a cofactor in catalytic activity of RNA polymerases and able to alter the expression of the genes through binding by compete with zinc in their site on transcription factor (Cousins, 1998). Increased embryonic silver accumulation may compete with zinc on the binding site or interact with thiol group of cysteine, histidine (C_2H_2) zinc finger protein causing inhibition of the catalytic function of zinc, thereby impair the gene expression. The decrease in *nkx2.5* expression in 72 h aged or hatched embryos in comparison with 24 h

aged embryos (Fig. 4.7B) confirmed that the activity of the gene at this age is not essential because the cardiac myocyte differentiation was completed.

In conclusion, the data obtained in the current study indicate the zebrafish early life stages are sensitive to waterborne silver. Twenty four hours aged live embryos accumulate more extent of silver than 72 h hatched embryos and 24 h dead one which start to show silver saturation. Live aged 24 h embryos also had higher K^+ and Na^+ concentrations than dead embryos and 72 h live hatched embryos. Silver toxicity is consistent with ionoregulatory toxicity although, there was no significant effect on Na^+K^+-ATPase activity. Silver exposure interferes with RNA-binding protein leading to impairment of the *nkx2.5* gene expression.

Chapter 5

Effect of waterborne silver on the protein expression profiles in early life stages (ELS) of the zebrafish (*Danio rerio*)

Effect of waterborne silver on the protein expression profiles in early life stages of the zebrafish (*Danio rerio*)

Abstract

Silver is widely distributed as a naturally occurring element, and its level increases due to anthropogenic activities such as silver mining, photographic processing, and through industrial and domestic wastewater effluents. Ionic silver (Ag^{+}) is highly toxic to aquatic organisms including fish and has a potential to cause developmental abnormalities when the exposure occurs in early life history stages. In the present study, proteomics analysis was performed to investigate changes in protein expression profiles in zebrafish embryos (<1 hpf) exposed (24 h) to 5 and 15 µg L^{-1} Ag^{+} as $AgNO_3$. Proteins extracted from homogenized embryos were analysed by mass spectrometry, and a total of 810 proteins and polypeptide were observed including induction of new proteins that are absent in control embryonic homogenate. Exposure to concentrations of 5 and 15 µg L^{-1} Ag caused induction of unique proteins that were not present in control embryonic homogenate. The analysis revealed that there was fold change of expressed proteins such as zona pellucida glycoprotein, ATP synthase subunit α and β, stressed proteins such as chaperone and heat shock proteins, antioxidant proteins such as CAT, SOD (Cu-Zn), and many enzymes like glutathione S-transferase M and glutathione S-transferase, in addition to proteins related to muscular development like myosin heavy polypeptide 2, actin alpha 1 skeletal muscle, slow myosin heavy chain 1, actin cytoplasmic 1, and tropomyosin.

5.1. Introduction

Proteomics assay has been used recently as a valuable biomarker to determine the toxicity of pollutants on the survival of the aquatic organisms (Shrader et al., 2003; Silvestre et al., 2006; Chora et al., 2009; Sanchez et al., 2009; Huang and Huang, 2012). The application of proteomics assay in zebrafish embryos leads to the identification of changes in the proteins during the exposure of embryos at early stages of embryonic development to the toxicants. Recently, several studies have highlighted the effect of toxicants on the early life stage of zebrafish (Shrader et al., 2003; Gündel et al., 2007; Shi et al., 2009).

Although there have been numerous studies on ecotoxicology of Ag in zebrafish, proteomics analyses have not been conducted previously. The aim of this study was to assess the effect of silver on the expression of proteins in zebrafish embryo during the segmentation stage (24 hpf). MaxQuant analysis proteomics analysis was used to investigate the differentiation of protein expression profiles of zebrafish embryos.

5.2. Hypothesis

In the previous experiment, the exposure of zebrafish embryos during the segmentation stage (24 hpf) to different concentrations of Ag showed disturbance in some biochemical assays and changes in the expression of *nkx2.5* and *mt2* genes. Now, the focus was on the compatible relationship between the proteomics assay and the biochemical assays, and if this would add new information about the effects of Ag during the embryonic development.

5.3. Material and methods

5.3.1. Experimental design and Preparation of embryos

Experimental design and Preparation of embryos were performed exactly as described in Chapter 4. Twenty fertilized embryos exposed to 0, 5, 15 µg L^{-1} Ag were collected in 1.5 Eppendorf tubes for proteomics analysis. Samples stored at -80 °C.

5.3.2. Extraction of protein

The extraction of 20 embryos was started by thawing and twice washing of embryos with phosphate-buffered saline (PBS) to remove any residues of the exposure solution. Sonication (Misonix, Microson, Ultrasonic Cell Disrupter, USA) was used to lysis the embryos in 300 µl of extraction buffer (7M urea, 2 thiourea, 4% CHAPS, 100 mM DTT, phosphate inhibitor mixture, complete protease inhibitor, Fisher Scientific UK). Each Eppendorf tube was placed in a 100 ml glass beaker filled with crushed ice, to keep the embryos sample cool during the sonication to avoid thermal denaturation of the protein. Prior to sonication, the sonicator probe was cleaned with 70% EtOH. The probe was inserted into the middle of the sample to insure good sonication. Sonication was performed for an initial 5 second burst. The bursts were set on level 2-3 on the instrument; then an additional period of sonication was applied to break any unbroken embryos which were still visible. All the samples were immediately stored in ice until the processing of other samples was completed.

5.3.3. Proteins separation by SDS-PAGE

To calibrate the abundance of the protein molecules of the samples, sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out. Briefly, due to the differentiation in the amount of protein in each sample, normalization with RIPA Lysis and Extraction Buffer (Sigma) was done to insure that the amount of the extracted protein was equal in each sample. Thirty

microlitres (contain 50 µg protein) of the normalized protein extraction of each sample in a 1 mL Eppendorf tube was mixed with 10 µl of dye and 2.1 µl of DTT (reducing agent). The mixture was centrifuged (Sigma, 1-14- Germany) at 11200 $_g$ for 30 s, and then all the samples were kept at 90 °C for 3 min on hot a plate (Strurat-Bibby, Scientific Limited-UK). The gel plate was left in an electrophoresis tank (BIO-RAD) which was filled with running buffer XT MES (Bio-Rad); each sample was loaded into the wells starting with ladder to identify the proteins bands. The electrophoresis tank was covered and connected to the power on 100 volts for 1.5 h. Then, the gel plate was stained with 10 µl of colloidal Coomassie blue stain (BIO-RAD) to visualise the protein band. The proteins bands were captured by scanning the gel plate (Epson, WF-3520).

5.3.4. Digestion of protein

5.3.4.1. Sample Preparation

The protein samples (30 µg) in urea buffer were digested following the Filter Aided Sample Preparation (FASP) methodology as described elsewhere (Wisniewski et al., 2009). The digested peptides were purified using the STAGE tip procedure as described previously (Juri Rappsilber, 2003). The protein digestion procedure was started by pre-wetting the membrane of the filter (Amicon Ultra-0.5 30 kDa Ultracel-PL memb 24/Pk) with 500 µl of 8 M urea buffer, then followed by centrifugation at 16200 g for 15 min. Thirty µl of the normalized protein extraction (contain 30 µg proteins) was added to the filter and the volume completed to the 500 µl mark with 8 M urea buffer, then centrifuge at 16200 *g* for 15 min. The flow through was discarded and 200 µl of 8 M urea buffer added to each filter and centrifuge at 16200 *g* for 15 min (another centrifugation was necessary to get as much of the urea through the filter as possible).

5.3.4.2. Reduction

Two µl of dithiothreitol (DDT) was added to the filter and the volume completed to 100 µl mark by the addition of 98 µl of urea buffer. All filters were incubated in a thermomixer at room temperature for 30 min, and then centrifuge at 16200 *g* for 15 min. To purify the sample, 400 µL of urea buffer was added for each filter and centrifuge at 16200 *g* for 15 min.

5.3.4.3. Alkylation with chloroacetamide

Ten µl of 500 mM chloroacetamide (47 mg chloroacetamide in 1 ml MQW) was added to the filter, and the volume completed to 100 µl mark, by the addition of 90 µl of urea buffer, centrifuge at 16200 *g* for 15 min. The filters were incubated at room temperature in a dark place for 20 min. Four hundred µl of urea buffer was added to the filters and centrifuge at 16200 *g* for 15 min. The flow through was discarded. The next step included the addition of 250 µl of ammonium bicarbonate buffer (ABC). One µg of trypsin (100 w/w, porcine trypsin, Promega) was added for each filter, and all the filters were kept at room temperature until the next morning. The spin filters were transferred to the new labelled centrifuge tubes, and centrifuge at 16200 *g* for 15 min. Fifty µl NaCl (500 mM) was added for the spin filter. The contents were mixed by shaking on a thermomixer (650 rpm) for 1 min, and then centrifuge for 30 min. The flow through was kept for the next step.

5.3.4.4. Acidification (to make the pH < 4)

To stop the reaction 50 µl of 2% trifluoroacetic acid (TFA) was added and mixed well with contents in room temperature by using the thermomixer (650 rpm) for 1 minute, then centrifuge at 16200 *g* for 15 min. pH papers (Fisher Scientific UK) were used to check that the pH of each sample was less than 4.

5.3.4.5. Purification

For the purification of protein, a Stage Tip procedure was done by inserting a small piece of filter into the narrow end of the tip. Then, this was followed by the activation of the stage tip with 50 µl of methanol (MeOH 100%), and the tip was subsequently centrifugation at 3500 rpm for 3 min. To ensure that the entire methanol had passed through the stage tip another centrifugation was done. The next step performed was the addition of 50 µl of buffer B to the stage tip, then another centrifuge at 4400 *g* for 3 min (centrifugation was repeated until all the volume has passed through the filter). Fifty µl of buffer A [0.5% acetic acid, 0.02% heptafluorobutyric acid (HFBA)] was added to stage tip and centrifuged at 4400 *g* for 3 min, another 50 µl of buffer A was added and centrifugation was repeated.

5.3.4.6. During and preservation of samples

One hundred µl (30 µg protein) of the sample was added to the stage tip carefully to avoid any disturbances of the particles and centrifugation at 3500 rpm / 900 *g* for 3 min was done and repeated until there was no sample left in the stage tip. The stage tip washed with 50 µl of buffer A and centrifuge at 4361 g for 3 min the flow through was discarded. The next step was the transferred of the stage tip to the new centrifuge tubes and eluted with 25 µl of buffer B (80% acetonitrile, 0.5% acetic acid, 0.02% HFBA), then centrifugation at 4400 *g* for 3 min. Another 25 µl of buffer B and centrifuge at 4400 *g* for 3 min. The last step included the concentration of the samples by using CentriVap (Concentrator plus) for 30 min. The last step included fast evaporation of the solvent, drying and concentration of the samples using a CentriVap (Concentrator plus) for 30 minutes. The samples were concentrated to a volume between 5-8 µl, and then transferred into glass Ms vials (Amicon®Ultra-Germany), and stored at -20 °C until ready for mass spectroscopy.

5.4. Mass Spectrometry Method

Peptides were separated on a Dionex Ultimate 3000 RSLC nano flow system (Dionex, Camberly UK). A 3 µl of sample was loaded in 0.1% trifluoroacetic acid (TFA) and acetonitrile (2% acetonitrile in 0.1% TFA) onto an Acclaim Pep Map100 µm × 2 cm, 3 µm C18 nano trap column, at a flow rate of 5 µl/min, by passing through the analytical column. Elution of bound peptides was performed with the trap column in line with an Acclaim PepMap C18 nano column 75 µm × 25 cm, 3 µm, 100 Å (Analytical Column) with a linear gradient of 96% buffer A and 4% buffer B to 60% buffer A and 40% buffer B, (Buffer A: 0.5% Acetic Acid, Buffer B: 80% acetonitrile in 0.5% acetic acid) at a constant flow rate of 300nl/min over 120 minutes. Each sample was ionized in positive ion mode using a Proxeon nano spray ESI source (Thermo Fisher Hemel UK) and analysed in an Orbitrap Velos Pro FTMS (Thermo Finnigan, Bremen, Germany). The Orbitrap Velos Pro instrument underXcalibur 2.1 software was operated in the data dependent mode to automatically switch between MS and MS/MS acquisition. MS spectra of intact peptides (m/z 350-1600) with an automated gain control accumulation target value of 1000000 ions were acquired with a resolution of 60000. The ten most intense ions were sequentially isolated and fragmented in the linear ion trap by collision induced dissociation (CID) at a target value of 10,000 or maximum ion time of 200 ms. A dynamic exclusion of ions previously sequenced within 45” was applied. All the singly charged and unassigned charge state ions were excluded from sequencing. Typical mass spectrometric conditions were: spray voltage, 2.3 kV; no sheath and auxiliary gas flow; heated capillary temperature, 275 °C; normalized CID collision energy 40% for MS2 in Linear Trap Quadropole (LTQ). The ion selection threshold was 10000 counts for MS2. An activation q = 0.25 and activation time of 30 ms were used.

5.5. Data Analysis by Max Quant 1.5.0.30

Peptides and proteins were identified by Andromeda via automated database searching of all tandem mass spectra against a curated target/decoy database (forward and reversed version of the *Danio rerio* sequence database (www.uniprot.org). Spectra were initially searched with a mass tolerance of 6 ppm in MS mode and 0.5 Da in MS/MS mode and strict trypsin specificity and allowing up to 3 missed cleavage sites. Cysteine carbamidomethylation was searched as a fixed modification, whereas N-acetyl protein, deamidated NQ, oxidized methionine were searched as variable modification. The resulting Andromeda peak list-output files were automatically loaded into inbuilt MaxQuant software modules for further processing and a maximum false discovery rate of 1% was fixed for the result output files. The proteomic data obtained was analysed using Perseus software provided with Max Quant software package.

The abundance of the proteins was calculated from the Normalised Intensity Values obtained after Max Quant analysis. The ratio of abundances was calculated to compare controls vs 5 µg L^{-1} silver treated and controls vs 15 µg L^{-1} silver treated groups. The experiment was done in biological replicates and a standard both sided *t*-test was performed to calculate the significance. The response is measured as number and type of proteins.

5.6. Results

5.6.1. SDS-PAGE

The result of SDS-PAGE confirmed the uniformity in extraction of protein from embryonic homogenates. The proteins appear as separated bands of the control and 5 and 15 µg L^{-1} Ag (Figure 5.1).

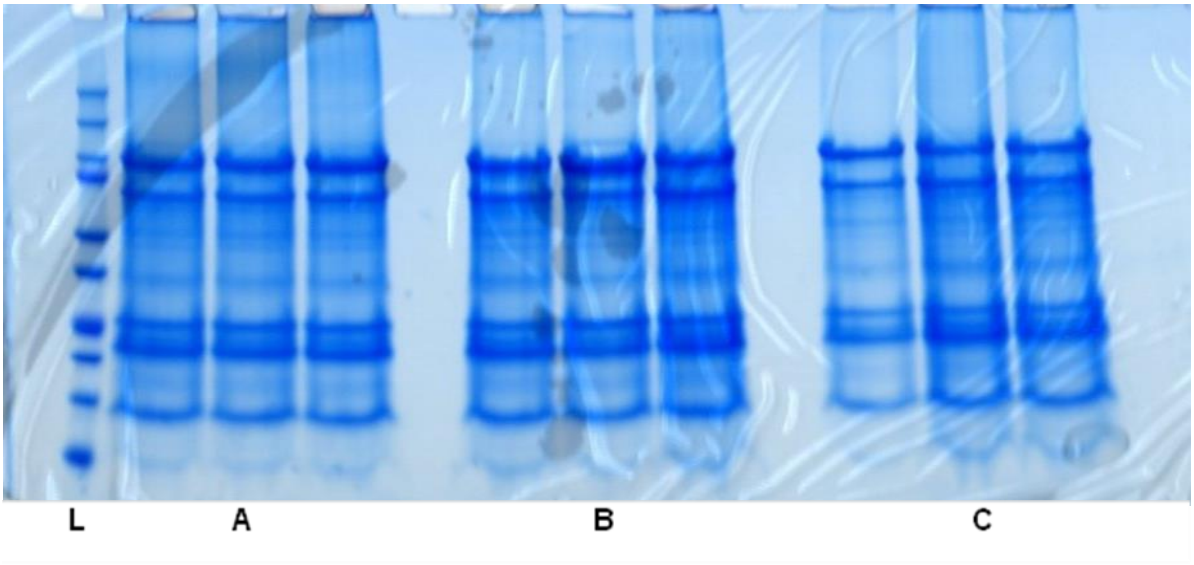

Figure 5.1 Separation of the embryonic homogenate proteins; control (A), 5 µg L^{-1} Ag (B), and 15 µg L^{-1} Ag (C), by SDS-PAGE, and visualized by Coomassie blue staining.

5.6.2. Mass spectrometry

Mass spectrometry identified a total of 810 proteins and some polypeptides in 24 hpf aged embryo samples and quantified changes in their abundance in response to silver treatment at two different doses (5 and 15 µg L^{-1}) further to the control. The total number of identified proteins included induction of 10 and 30 respectively for the Ag exposures, as newly expressed proteins which were

absent in the control y (Figure 5.3). The Label-Free Quantification (LFQ) method in mass spectrometry was used to determine the relative amount of proteins in the embryonic homogenate. The LFQ intensity correlation plot between biological replicates was revealed using scatter plots of identified proteins of the embryonic homogenate of control and silver exposure groups. Each dot represents a single protein identified in each treatment (Figure 5.2). The MS analysis of 810 protein spots expressed during the segmentation stage (24 hpf) showed changes in the protein expression profiles in embryonic homogenate that coincided with silver exposure.

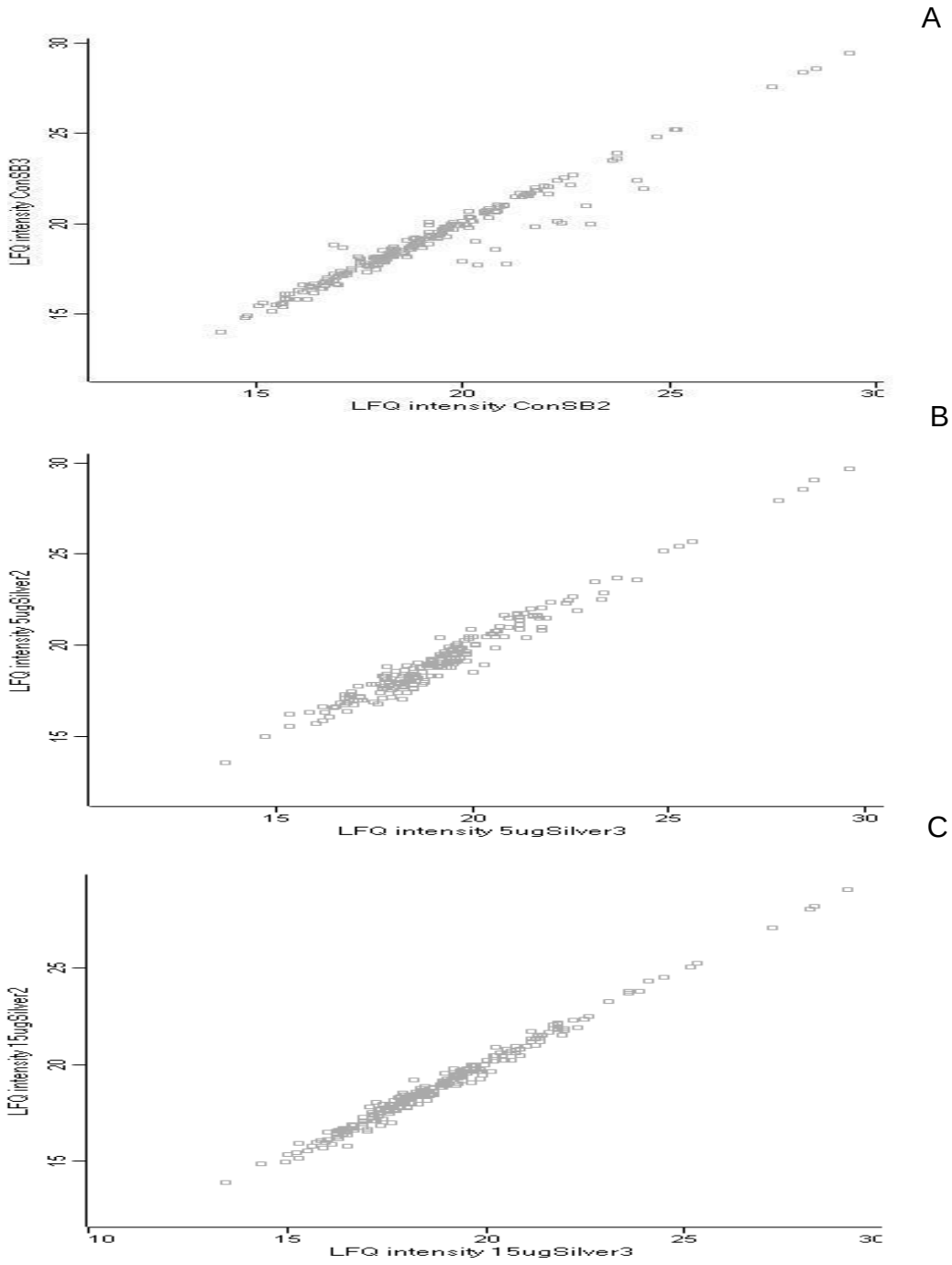

Figure 5.2 Scatter plots of LFQ replicates (*n* = 3) of identified proteins of control (A), 5 µg L^{-1} Ag (B), and 15 µg L^{-1} Ag (C).

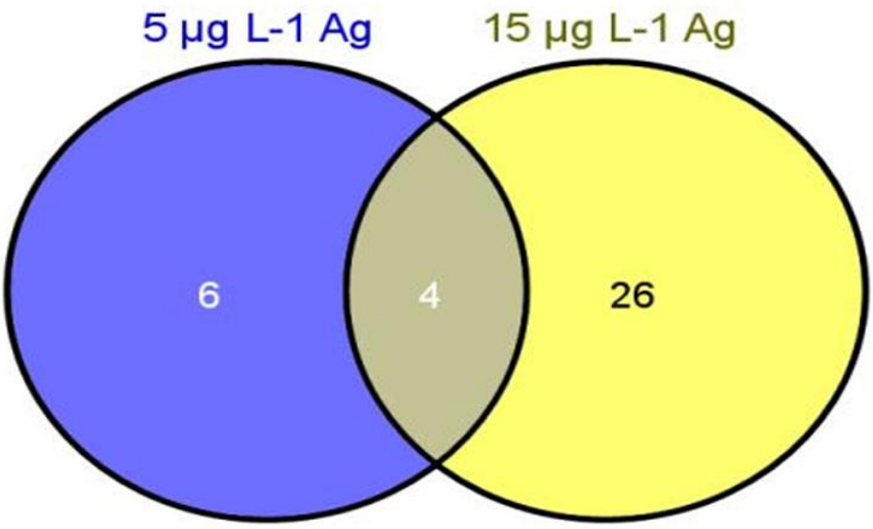

Figure 5.3 Venn diagram showing the number of expressed proteins induced due to the exposure to 5 and 15 µg Ag L^{-1}.and absent in control. Numbers that fall into more than one circle were common to both concentrations, whereas numbers in only one circle indicate the expressed proteins that were unique for each concentration.

Mass spectrometry analysis of 810 expressed proteins and polypeptides showed there were 249 embryonic homogenate proteins and poly peptides that showed fold changes during the MS analysis. Thirty two of the 249 expressed proteins and polypeptides (Table 5.1, Fig. 5.4) were selected according to the result of the experiment of silver exposure (Chapter 4) revealed variation in fold changes (increase or decrease) due to exposure to silver concentrations (5 and 15 µg L^{-1}). Among 32 expressed proteins, the result of the proteomics analysis of embryonic homogenate of embryos exposed to silver concentrations showed there were increased fold change of expressed proteins related to non-exposed embryos. Zona pellucida glycoproteins (Fig. 5.5) increased as silver concentrations increased. Despite this observation, there were no delays in growth of embryos, but muscular development proteins like myosin heavy polypeptide 2, actin alpha 1 skeletal muscle, and slow myosin heavy chain 1 showed fold decreases as silver concentrations increased. In contrast, tropomyosin proteins, myosin light chain alkali smooth muscle isoform and actin alpha cardiac muscle 1a showed fold increases (Fig. 5.6). Variable fold change was seen in stress proteins such as heat shock protein and chaperonin (Fig.5.7). The analysis showed there were increased fold changes as silver concentrations increased in antioxidant proteins such as catalase (CAT), superoxide dismutase (Cu-Zn), glutathione S-transferase M, glutathione S-transferase, and L-lactate dehydrogenase B-A chain (Fig. 5.8). Fold increases in ATP synthase subunit α and β were observed as silver concentrations increased (Fig. 5.9). The proteomics analysis did not show detection of cardiac proteins and metallothionein. The result of proteomics analysis showed there were presence of large number of uncharacterized proteins, more analytic technique such as western blotting is needed to detect specific proteins in the embryonic homogenate such as cardiac proteins and metallothionein.

Table 5.1 Name and function of selected expressed proteins of embryos exposed to silver concentrations 5 and 15 µg L^{-1} for 24 h

Protein names	Protein function
zona pellucida-like domains	Serve as structural components of egg coats.
Zona pellucida glycoprotein 3.2	Surrounds the oocyte and early embryo and serves as an important regulator of fertilization.
Chaperone protein GP96	Protect proteins against heat stress and other stress condition.
Chaperonin-containing TCP-1 complex beta chain (Fragment)	
Chaperonin containing TCP1, subunit 5	
Chaperonin containing TCP1, subunit 7 (Eta)	
Chaperonin containing TCP1, subunit 6A (Zeta 1)	
Heat shock 60kD protein 1 (Chaperonin	
Heat shock protein 5	
Heat shock protein 9	
Heat shock 70kDa protein 8	
Heat shock protein 25	
Heat shock protein 90-alpha 2	
Protein disulfide-isomerase	Valuable marker in fish exposed to the environmental stress (stress proteins).
Protein disulfide-isomerase (Fragment)	
Protein disulfide isomerase-related protein P5	
Actin, cytoplasmic 1	Involved in various types of cell motility and expressed in all eukaryotic cells. Providing mechanical strength to the cell membrane.
Actin, alpha 1, skeletal muscle	Involved in cell motility, structure and integrity.

Actin, alpha, cardiac muscle 1a	Major constituent of the contractile apparatus are involved in various types of cell motility.
Slow myosin heavy chain 1	Expressed primarily in the heart and also in skeletal muscles and plays a major role in cardiac muscle contraction.
Myosin light chain alkali, smooth-muscle isoform	Structural component of muscle regulate muscle contraction.
ATP synthase subunit alpha	Transfer of a substance from one side of a cell membrane to the other.
ATP synthase subunit beta	Catalysis of the transfer of hydrogen ions from one side of a membrane to the other.
Na^+/K^+ ATPase alpha subunit isoform 8	An integral membrane protein responsible for maintaining the electrochemical gradients of Na and K ions across the plasma membrane.
ATP synthase, H^+ transporting, mitochondrial F0 complex, subunit d	Catalysis the transfer of hydrogen ions from one side of a membrane to the other.
Calcium-transporting ATPase	It is vital for regulating the amount of Ca^{2+} within cells.
Superoxide dismutase [Cu-Zn]	An important antioxidant defense in living cells exposed to oxygen. Catalyse the dismutation (or partitioning) of the superoxide (O_2^-) radical into either ordinary molecular oxygen (O_2) or hydrogen peroxide (H_2O_2).
Glutathione S-transferase	Play an important role in detoxification by catalysing the conjugation of many hydrophobic and electrophilic compounds with reduced glutathione.
Glutathione S-transferase M	Catalyse metabolic pathways for the excretion of reactive oxygen species that may be generated by cellular oxidative stress.

Catalase (Fragment)	It catalyses the decomposition of H_2O_2 to water and oxygen. Protect the cell from oxidative damage by reactive oxygen species (ROS).
L-lactate dehydrogenase B-A chain	Released during tissue damage, it is a marker of common injuries and disease.

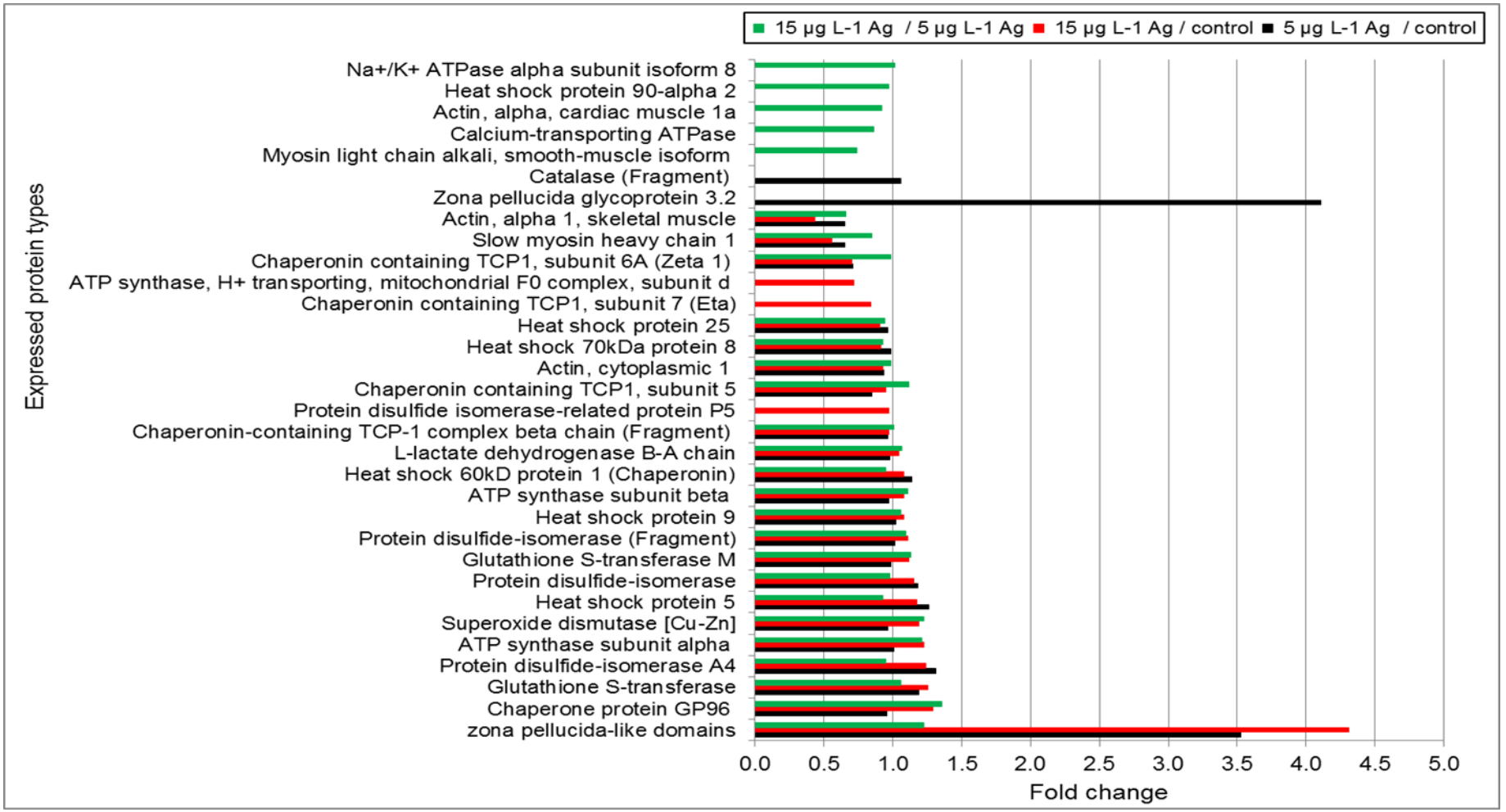

Figure 5.4 Fold changes of selected expressed proteins of embryos exposed to concentrations of 5 and 15 µg Ag L^{-1} related to the control. The number of live embryos analysed for each condition was (20). Data are means ± S.E.M., *n* = 3 samples pre-treatment.

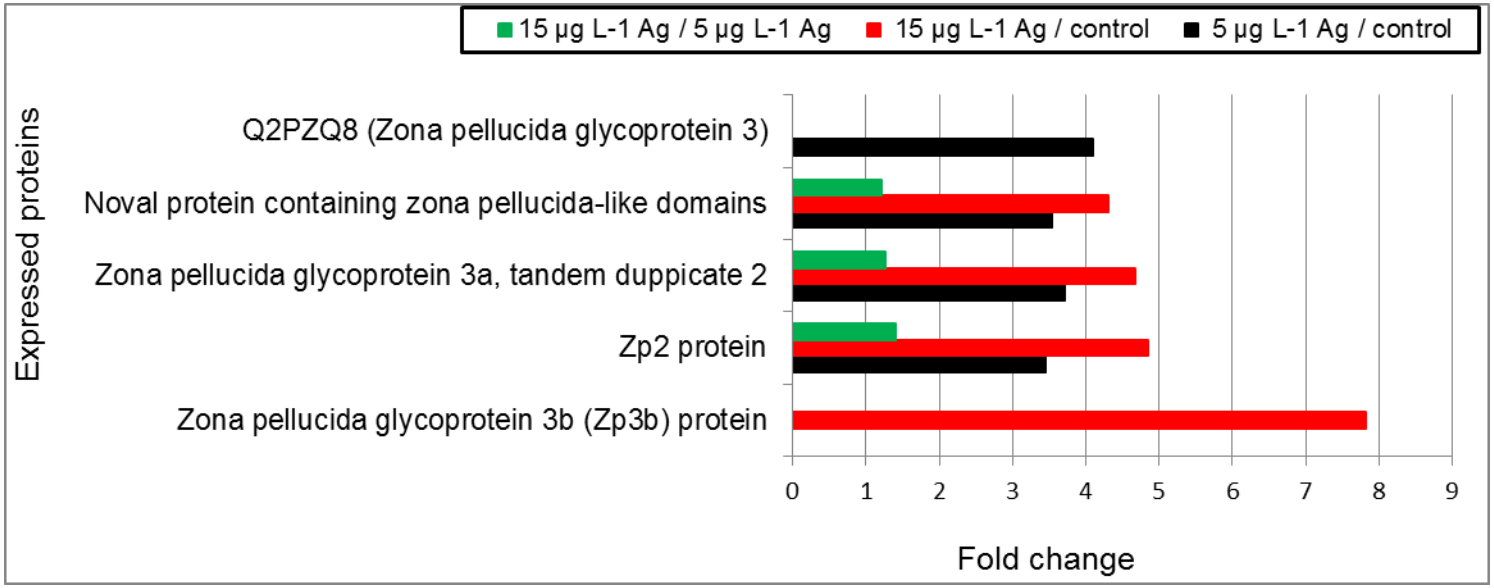

Figure 5.5 Fold changes of expressed zona pellucida glycoprotein protein related to embryos exposed to 5 and 15 µg Ag L^{-1}. The number of analysed live embryos was 20. Data are mean ± S.E.M., *n* = 3 samples per treatment

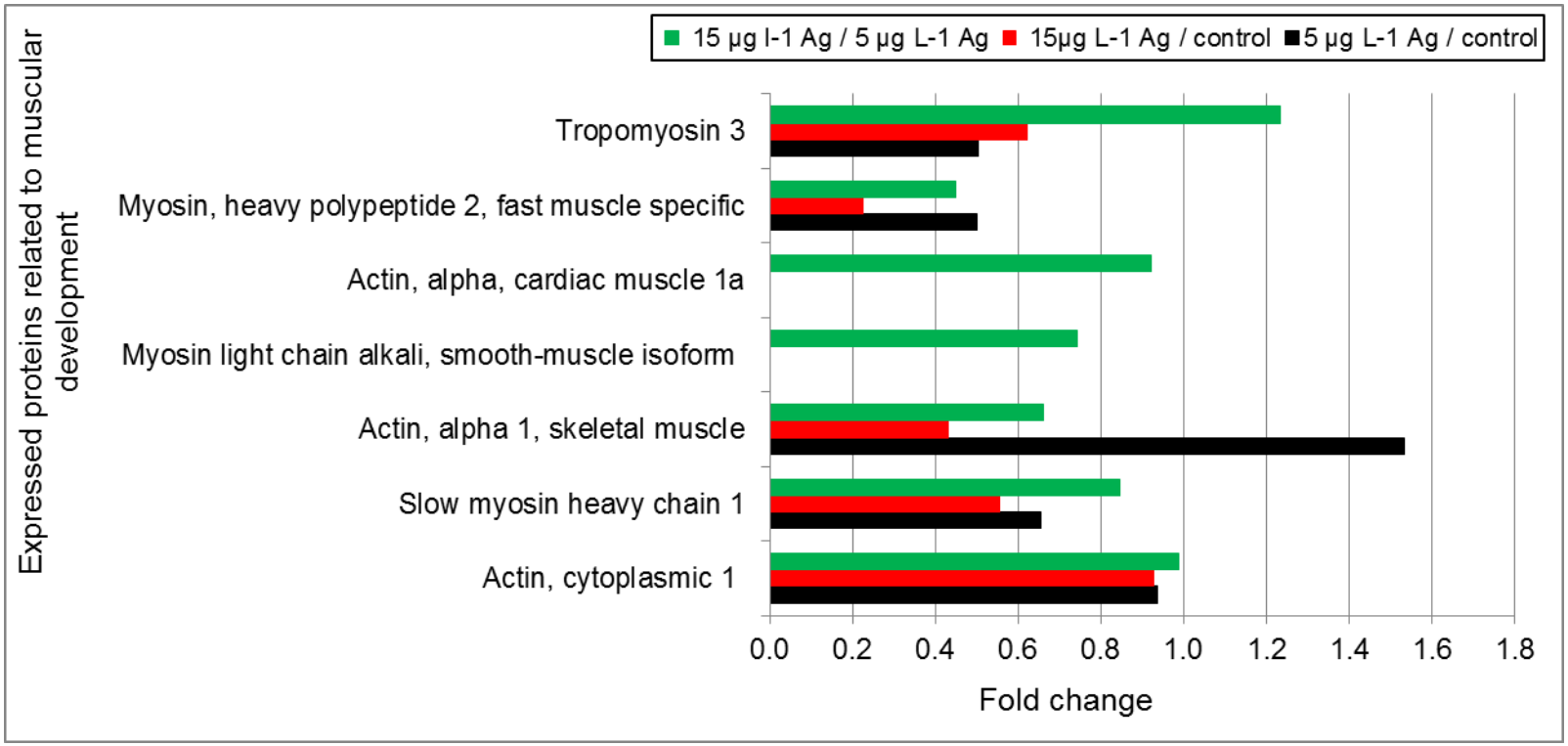

Figure 5.6 Fold change of expressed protein related to muscular development of embryos exposed to concentrations of 5 and 15 µg Ag L^{-1}. The number of live embryos analysed for each condition was (20). Data are means ± S.E.M., *n* = 3 samples per treatment.

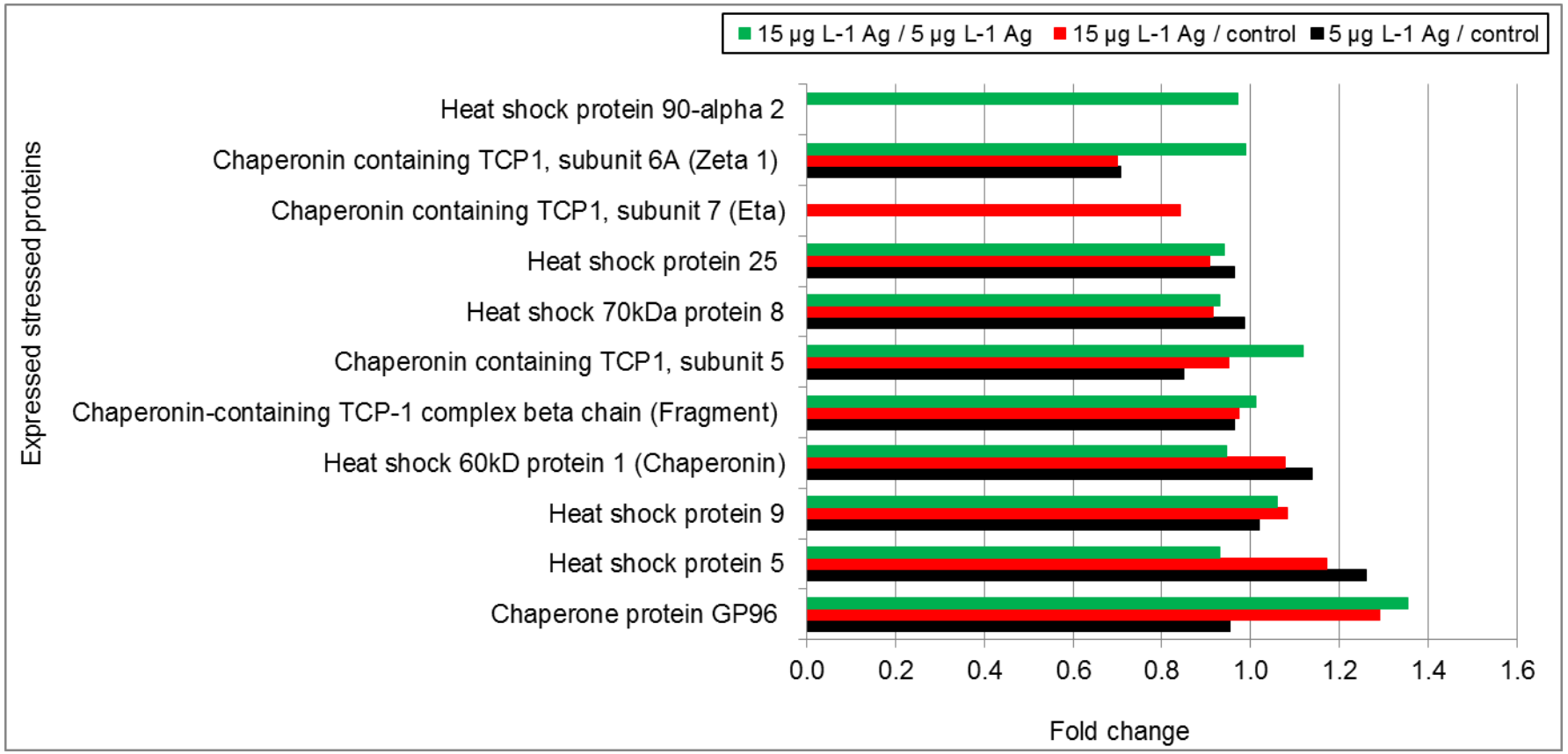

Figure 5.7 Fold changes of expressed stress proteins of embryos exposed to concentrations of 5 and 15 µg Ag L^{-1}. The number of live embryos analysed for each condition was (20). Data are means ± S.E.M., *n* = 3 samples per treatment.

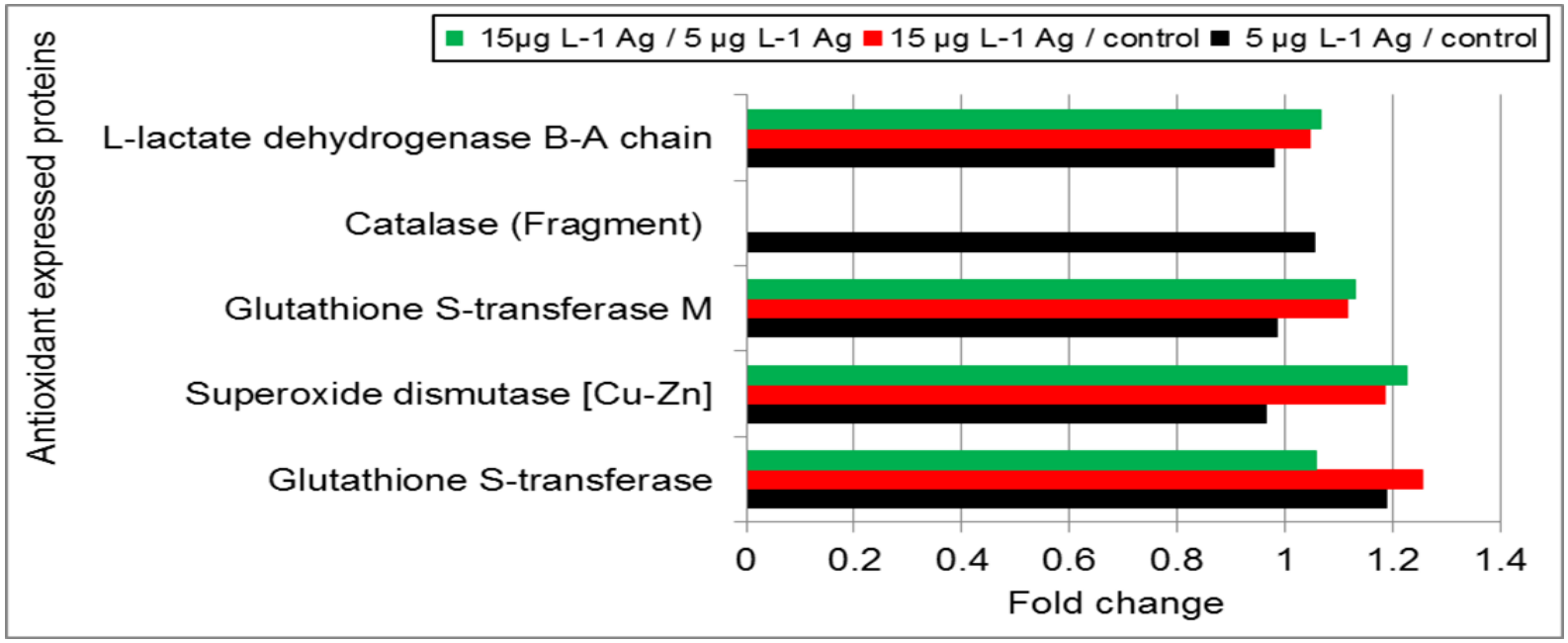

Figure 5.8 Fold changes of antioxidant expressed proteins and L-lactate dehydrogenase B-A chain of embryos exposed to concentrations of 5 and 15 µg Ag L^{-1}. The number of live embryos analysed for each condition was (20). Data are means ± S.E.M., *n* = 3 samples per treatment.

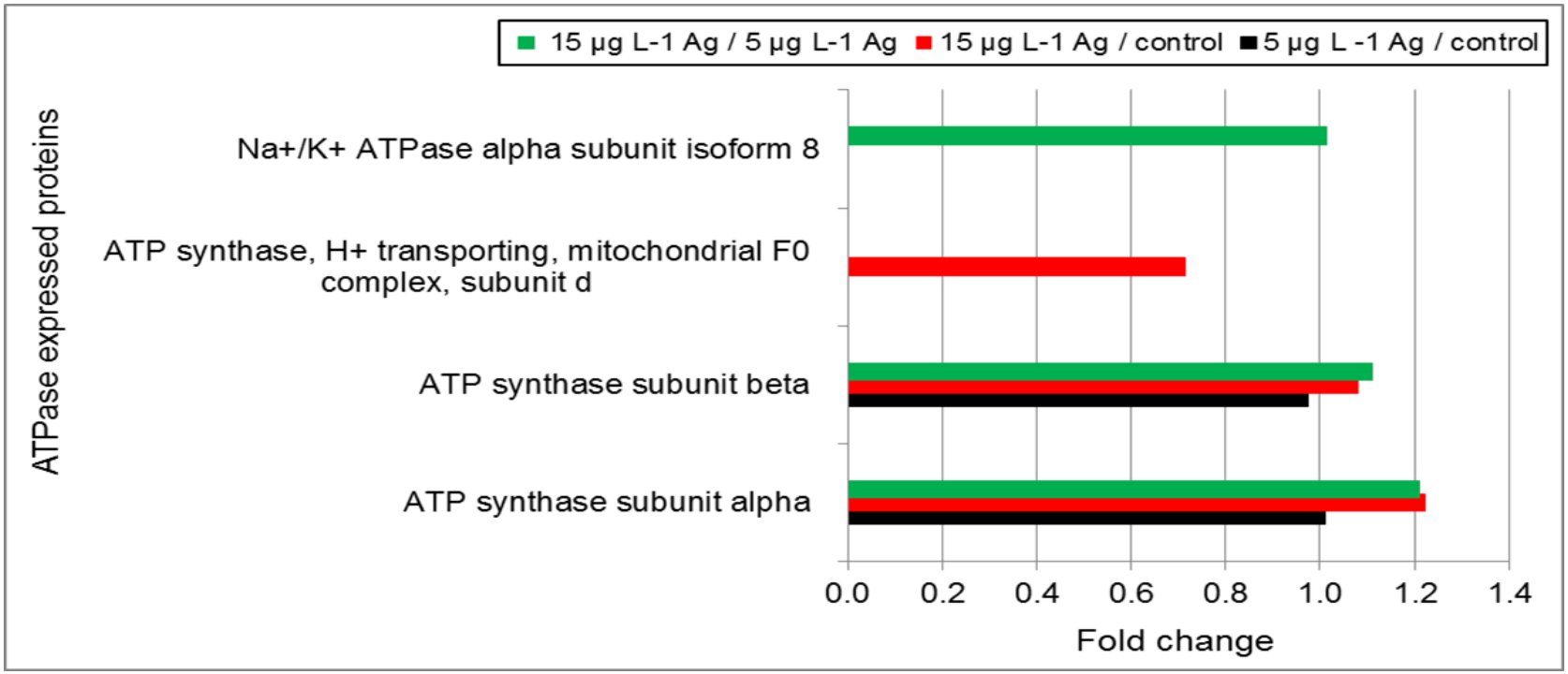

Figure 5.9 Fold change of ATPase expressed proteins of embryos exposed to concentrations of 5 and 15 µg Ag L^{-1}. The number of live embryos analysed for each condition was (20). Data are means ± S.E.M., *n* = 3 samples per treatment.

5.7. Discussion

To our knowledge, the current experiment is the first to investigate the effect of waterborne silver on the expression of proteins in the early life stage of zebrafish. The MS analysis of 810 protein spots expressed during the segmentation stage (24 hpf) confirms the requirement of these proteins to maintain normal organogenesis and thereby, normal growth of the embryo. The exposure of < 1 hpf embryos to silver for 24 hpf caused changes in protein expression profiles (PEPs) that coincided with increased embryonic silver accumulation. The expression of the large number of proteins is considered as a requirement for the development of the embryo and also to maintain its normal growth.

Embryo exposed to silver for 24 h showed induction of new expressed proteins with silver concentrations increase (Fig. 5.3). The majority of new induced proteins are non-characterized, and their number increased in embryos exposed to 15 µg L^{-1} than those exposed to 5 µg L^{-1}. Among the 249 expressed proteins, 32 expressed proteins (Fig. 5.4) were selected to show the effect of silver exposure and if there is compatibility between biochemical and proteomics assays.

Zona pellucida glycoprotein types (Fig. 5.5) were identified and showed an increase in fold change as silver concentrations increased related to control. Zona pellucida glycoproteins are characterized by their ability to act as sperm receptors and are essential to initiate normal fertilization (Wassarman, 2002; 2008). They are also considered as a trap or site of binding with metal cations due to the presence of −SH groups as constituents of glycoproteins (Rombough, 1985; Sugiyama et al., 1996).

Among the expressed proteins, there were a number of proteins involved in muscular development (Fig. 5.6). Although there were increased fold changes relative to the control, decrease in fold change was seen in embryos exposed to

15 µg L^{-1}. The identification and increased fold of myosin heavy polypeptide 2, actin alpha 1 skeletal muscle, slow myosin heavy chain 1, actin cytoplasmic 1, and tropomyosin proteins that are related to the structure and function of cytoskeleton indicate the requirement of these proteins for the normal embryonic development at early stage of development and may substitute the damaged proteins due to the toxic effect of silver. The embryos exposed to silver in the main experiment (chapter 4) did not show any retardation or delay in growth. It was hypothesised by Rodríguez-Ortega et al. (2003) that the damaged cytoskeletal proteins in the bivalve mollusc (*Chamaelea gallina*) exposed to the environmental pollutants increased the expression of protein, which is related to the structure and function of cytoskeleton to assist the normal growth and development.

As a result of silver exposure, exposed embryos showed increase in fold of stress proteins such as heat shock protein and chaperonin (Fig. 5.7) relative to the control. These proteins are considered as protective proteins against the elevation of temperature as well as toxicants (Lutsch et al., 1997; Choi et al., 2008). Exposure to silver may promote the production of the stress proteins to avoid any disturbances in the mechanism of protein synthesis that leads to the damage of newly protein production (Kalmar and Greensmith, 2009).

The previous experiment (chapter 4) showed no effect of silver on total GSH level in 24 h live embryo, although the proteomics analysis revealed that silver exposure elicits the production of GSH transferase. On the other hand, there was induction of antioxidant enzymes such as CAT and SOD (Cu-Zn) as an indicator of levels of ROS despite the concentration of silver and the period of exposure being short, and there was no evidence of generation of ROS that leads to OS in embryos (Griffitt et al., 2009). On the other hand, L-lactate dehydrogenase B-A chain in the silver-exposed embryos showed an increase fold related to the control. Such increase may indicate the presence of physiological disturbance due to the damage of embryonic tissues. The elevation in lactate dehydrogenase (LDH) activity as a cytoplasmic enzyme was observed in the damaged muscle and hepatopancreas tissues of *Sesarma*

quadratum exposed to copper chloride (Valarmathi and Azariah, 2003) and in the gill epithelium of *Channa punctatus* exposed to cadmium and copper (Sastry et al., 1997). The distortions in the cell organelle such as mitochondrial damage cause reduction in O_2 uptake capacity and hypoxia of the organ.

Exposure of embryos to silver showed an increase in ATP synthase subunit α and subunit β relative to the control (Fig. 5.9). The increased embryonic silver concentrations as seen in the previous experiment promote the production of ATPase subunits to substitute the inactive ATPase proteins due to silver toxicity. It is well known in adult fish that Ag is a rapid and potent inhibitor of Na^+K^+-ATPase activity due to its ability to interact with −SH groups of the Na^+K^+-ATPase enzyme (Rombough, 1985; Hogstrand and Wood, 1998).

In conclusion, proteomics analysis has shown to be a valuable assay to investigate the toxic effects of silver in aquatic organisms. Mass spectrometry analysis successfully identified a total of 810 proteins in 24 hpf aged embryo. Overall, through the results of the current study, it appeared that silver exposure induces several changes in PEPs in zebrafish embryos. Silver exposure also increases the promotion of the production of proteins that are essential for the growth and maintenance of the embryo, particularly during the early stage of the development. To our knowledge, this is the first study that indicates the possibility to use the zebrafish embryo proteomics as a useful tool for investigation of the environmental risk that is caused by heavy metals or other pollutants.

Chapter 6

General discussion

6. General discussion

The study gives a new understanding of copper and silver toxicity on some biochemical and molecular processes that are necessary to maintain the normal development and growth of embryos, particularly during the organogenesis stage. The results of the current experiments are mostly consistent with our hypothesis, while other results seem incompatible with expectations of the hypothesis (Fig 1.2). Figure 6.1 summarizes the results of the current experiments.

Disturbances in Na^+K^+-ATPase activity and electrolyte imbalance represent the main changes in the biochemical process in embryos exposed to Cu and Ag. Copper and Ag as transition metals have high affinity to bind avidly to the –SH groups of Na^+K^+-ATPase molecules and mucoproteins of PVF. A low numbers of under developed ionocytes as well as the exposure concentrations of trace metals may play an important role to determine the efficiency of osmoregulatory system and balance of electrolyte. Exposure of < 1 hpf embryos to copper concentrations for 16 hpf (Chapter 3) showed impairment of Na^+K^+-ATPase and electrolyte imbalance as the main effect of Cu exposure. In adult fish, Na^+K^+-ATPase is the main enzyme affected by Ag and considered as the key mechanism of osmoregulation. Exposure of embryos to Ag for 24 h and 72 h did not significantly affect the activity of Na^+K^+-ATPase. On the other hand, unexposed 72 h hatched embryos showed increased in the activity of Na^+K^+-ATPase in comparison with unexposed 24 h aged embryos, probably due to the development of osmoregulatory organs such as gills in hatched embryos.

Embryos were showed significant increase in *Mt2* expression during exposure to Cu without added Ca. Increased heartbeats were seen in embryos exposed to Cu and Ag probably relate to Na^+ pump inhibition and a subsequent Ca^{2+} influx on the Na^+/Ca^{2+} exchanger that leads to increased depolarisation of the sarcolemma (Chapters 3 and 4).

The proteomics assay is of value as a complementary assay to the biochemical and gene transcription assays. When the biochemical assays determine the activity of biochemical reaction, proteomics assays give an induction about the type and concentration of the protein that is necessary to achieve the biochemical reaction. Due to the lack of information about the effect of silver on protein expression profile during embryonic development, proteomics analysis was used to investigate the changes in the expressed proteins. Proteomics analysis is of value in ecotoxicology to investigate the up and down regulated proteins as a probable target of the toxic metals like silver. The expression of 810 proteins confirms the requirement of the embryos at organogenesis stage for a high level of expressed proteins to maintain the normal development and growth particularly during the organogenesis stage (Chapter 5).

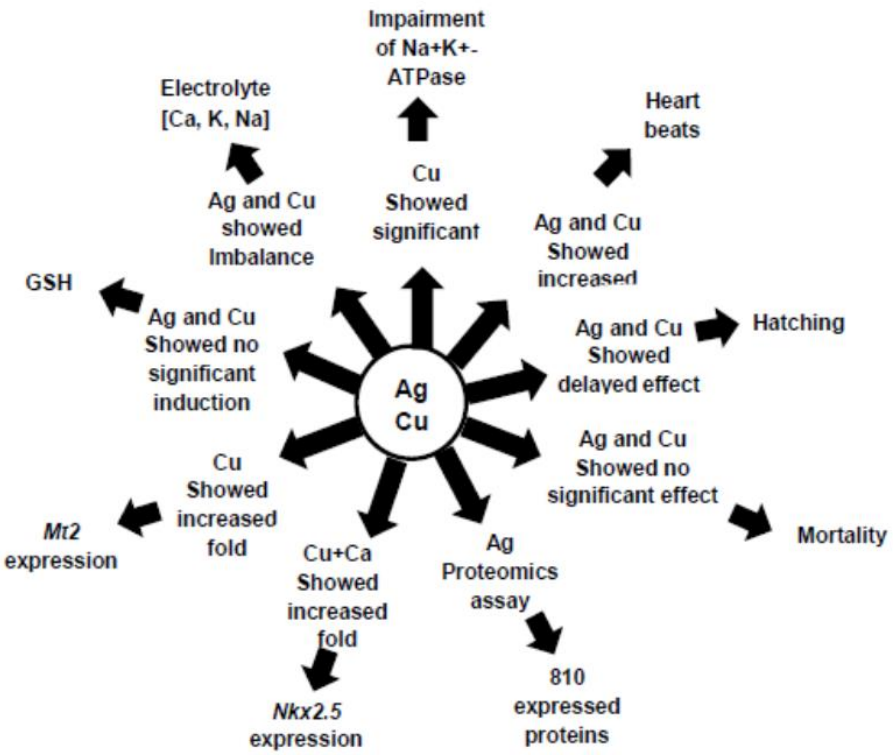

Figure 6.1 Schematic diagram showed the result of the current experiments.

6.1. Effect of copper and silver on the early life stage (ELS)

Exposure of embryos (< 1 hpf) in the current experiment to a high concentration of Cu as an essential trace metal micronutrient and Ag as a non-essential trace metal, earlier after fertilization and before the hardening of the chorion initiates an adverse effect on the survival of the embryo. At the early stage of embryonic development, the embryo is more sensitive and vulnerable to the toxic concentrations of metals. In the current experiment, embryos showed increased accumulation of Cu and Ag as the exposure concentrations were elevated, confirming a link between exposure and effect due to internalisation of metals in the embryo. Mortality of embryos increased significantly as copper concentrations increased, while exposure of embryos to Ag concentrations did not show significant effect on mortality even though Ag is one of the highly non-essential toxic metals. The non-significant mortality in Ag experiments may be explained as a result of low nominal concentration of Ag that was used in the current experiments. On the other hand, the presence of maternal metallothionein may be interfering with accumulated embryonic Ag. Higher mortality during exposure of embryos to Cu concentrations was observed at the blastula and gastrula stages particularly within first 10 hpf; which is considered as the more vulnerable stage and the time when hardening of the chorion occurs (Weis and Weis, 1991). Higher mortality was observed in embryos exposed to Cu during first 10 h (1–10 h) and continuously (1–72 h), in comparison with the low mortality in unexposed (control) and exposed embryos to Cu between 10–72 hpf. This confirms that the first 10 h are the most vulnerable and sensitive stage during the development of the embryo (Chapter 3).

Passive entrance plays an important role in the transfer of Cu and Ca into embryo from the surroundings. Live and dead embryos exposed to Cu with and without added Ca showed increased Cu concentrations coinciding with increased Ca concentrations. The increased embryonic Cu and Ca concentrations were more pronounced in dead than live embryos exposed to Cu with added Ca, which resulted due to the passive entrance of Cu and Ca

from the surroundings. In the current study (Chapter 3), embryos were exposed to two concentrations of Ca^{2+}, 20.91 mg L^{-1} as the background with no further added Ca^{2+} and added 40 mg L^{-1} as $CaCl_2.2H_2O$, which represents two different levels of hardness. Addition of Ca (Chapter 3) appeared to ameliorate copper toxicity in the embryos even though there was an increase of embryonic copper concentrations. As Ca concentrations increased in the exposure solutions, there were also elevations in embryonic Ca levels. One explanation of this observation is that Ca passively increased filling the anionic residues in the PVF (simple ion exchange for ions such as H^+, Na^+, and K^+ in the PVF). Once the passive potential becomes less negative, zero, or positive, the passive Cu entry will go down (Shephard, 1987). The presence of embryonic Ca will compete with Cu for the binding with –SH groups in the mucoproteins of the PVF, leading to reduction in Cu toxicity. The effect of added Ca on passive potentials across the chorion may protect against increasing free metal ion activity of Cu in the embryo.

On the other hand, live embryos that were exposed to silver for 24 h showed increased embryonic Ag concentrations more than the 24 h dead and 72 h aged (hatched embryos) embryos (Chapter 4). The passive entrance of Ag as a metal cation, like the entrance of Ca and Cu, depends on simple ion exchange until saturation of the anionic residues occurs in the PVF with Ag ions. The passive entrance of Ag will go down when the passive potential becomes zero or positive. Further for the passive movement of water and ions, the chorion plays a role in the transport of metal ions from the ambient to the embryo particularly during the swelling phase of egg shell (chorion). The presence of mucoproteins as a component of the chorionic protein constituent acts as a trap for the metal cations (positive charge). Cu and Ag ions bind avidly to −SH group in the mucoproteins of the PVF leading to disturbance in the exchange of metal cations between perivitelline spaces and ambient. Increased embryonic Cu and Ag concentrations as the result of their exposure coincided with K^+ and Na^+ depletion in dead embryos exposed to Cu and Ag and hatched embryos exposed to Ag, which probably occurred due to the ion exchange.

Increased Cu and Ag exposure causes decreased and delayed hatching, although Ag did not show adverse effect on mortality of embryos. It is probable that Cu and Ag can impair the activity of chorionase as a disintegrator of the zona interna of the chorion through the interaction with their −SH group. The increased copper and silver embryonic concentration as seen in current experiment (Chapter 3 and 4) increased the probability of the interaction with disulphide bonds of the chorionase that causes disturbance in enzyme activity. On the other hand, Cu and Ag can bind with the DNA, causing possible damage of DNA due to the generation of free radicals which lead to the failure in transcription and expression of proteins.

6.2. Effect of Cu and Ag on Na^+K^+-ATPase as an ionoregulatory membrane transporter

Impairment of Na^+K^+-ATPase activity was seen in embryos exposed to copper without added calcium, while the presence of high embryonic Ca concentrations antagonized Cu toxicity. In contrast, the nominal silver concentrations used in the experiment (Chapter 4) did not show a significant effect on the activity of Na^+K^+-ATPase in 24 h aged embryos and hatched embryos, although the activity of Na^+K^+-ATPase was more pronounced in hatched embryos than 24 h aged embryos. The low concentrations of Ag may explain the failure of Ag to initiate the impairment of Na^+K^+-ATPase activity. Both Cu and Ag^+ compete with Mg^{2+} on the binding sites on the α subunit of the Na^+K^+-ATPase molecule that prevents the hydrolysis of ATP and liberation of inorganic Pi as a necessary step in the activation of Na^+K^+-ATPase.

Number, location, and well-developed ionocytes that are abundant with Na^+K^+-ATPase play an important role to control the osmoregulation. Embryos during the early stages of development particularly when the osmoregulatory organs such as gill and kidney are absent or not well developed. The presence of ionocyte (chloride cells) on the yolk sac membrane and body skin tegument is considered as a substitute of osmoregulatory organs (Tytler et al., 1993; Kaneko et al., 2002; Varsamos et al., 2002; Sucré et al., 2010). The impairment

of Na^+K^+-ATPase activity was observed in 16 h aged embryos exposed to Cu without added Ca in comparison to unexposed embryos. It may also explain the failure of increased Cu concentration to show significant effect on the Na^+K^+-ATPase activity in exposed embryos to Cu without added Ca, or Cu may interact with the limited number of Na^+K^+-ATPase molecules, and the increased Cu concentrations did not show an increase in the impairment of Na^+K^+-ATPase activity. On the other hand, increased Cu concentrations may be consumed to meet the requirement of biochemical processes during the embryonic development stages.

6.3. Role of metallothionein and glutathione in copper and silver toxicity

It was suggested in previous studies that the threshold of essential and non-essential metals must be enough to trigger the induction of GSH and MT (Atli and Canli, 2008). The result of the experiment showed that the concentrations of Cu used in the experiment are enough to initiate the significant effect on the expression of *Mt2* gene, whereas they are not enough for GSH induction. In contrast, exposure concentration of Ag, as a non-essential metal, is not enough for the induction of significant effect on GSH and *Mt2* expression gene.

Metallothionein and GSH play a role in the metabolism and homeostasis of low concentrations of Cu as well as detoxification of toxic concentrations of Cu and other metals such as Ag. The increased Cu and Ag embryonic concentrations as a result of increased exposure concentrations did not show a significant effect on GSH level, although GSH is considered as the first line of cellular defence against metal exposure. The proteomics analysis of embryonic homogenate of exposed embryos to Ag showed increase fold in SOD and CAT as antioxidant enzymes, which may confirm the occurrence of oxidative stress. Copper and Ag possess high affinity to interact with GSH –SH group and form stable complexes. These complexes may result in unchanged level of GSH as shown in embryos exposed to Cu with and without added Ca (Chapter 3) and Ag (Chapter 4). However, GSH is a Cu carrier; it conjugates with Cu forming a

Cu-GSH complex that acts as a donor of Cu ion to form Cu/Zn SOD (Ferreira et al., 1993). The increased embryonic Cu concentrations may cause increase in the formation of SOD as an antioxidant enzyme (not determined in our experiment). The interaction between the Cu and Ag with GSH and formation of Cu-GSH and Ag-GSH complexes may explain the low level of embryonic GSH. On the other hand, Cu and Ag may affect the essential enzyme directly that assists in the synthesis of GSH, such as GSH synthetase and GSH reductase. Previous studies showed that Cu inhibits the activity of GSH synthetase (Canesi et al., 1999). In contrast, the proteomics analysis of the embryonic homogenate of Ag-exposed embryos (Chapter 5) showed increase in induction of GSH transferase versus the non-significant effect of Ag on GSH level (Chapter 4). The increase in the level of GST may be considered as a reflex of decreased or unchangeable cellular GSH content (Canesi et al., 1999).

Exposure of the embryos to copper without added calcium in the current experiment (Chapter 3) showed increased *mt2* expression, which was concomitant with increased embryonic copper accumulation. Copper is capable to potentiate the induction of *mt2* expression, although studies have observed high accumulation of maternal metallothionein (Riggio et al., 2003). Increased embryonic Cu concentration as an activator of Zn finger proteins triggers the activation of transcription of metal-regulatory transcription factor-1 (MTF-1) as an initial step in the induction of *mt2* expression. The interaction with Zn finger cysteine –SH groups synergizes the activation of Zn finger protein for the binding between MTF-1 and MREs (Heuchel et al., 1994; Andrews, 2001; Craig et al., 2009),whereas the addition of Ca interferes with Cu and causes displacement of Cu for binding with Zn finger protein that leads to decrease in *mt2* expression.

In contrast, increased accumulation of embryonic Ag did not trigger the expression of *mt2* gene as in Cu exposure, although there was an increased embryonic Ag concentration. Ag like Cu has affinity to interact with Zn finger protein, but the nominal concentration of Ag that was used in the current experiment may be not enough to trigger the induction of metallothionein.

6.4. Do copper and silver effect the formation and development of the heart?

The heart is the first organ of the cardiovascular system that forms and functions in the embryo. It appears more vulnerable and sensitive to trace metal toxicity particularly during the organogenesis stage (Glickman and Yelon, 2002; Li et al., 2009; Barjhoux et al., 2012). Cu is an essential metal required to maintain the normal activity of cardiac muscle. The heart is recognized by the presence of high levels of GSH that act as a carrier of Cu. Exposure of embryos to Ag and high concentration of Cu continuously (1–72 h) increased the heartbeats that were recorded at 36 hpf. The elevation in heart rate might be a response to the toxic effect of trace metals as in adult fish. An embryo is able to cope with disturbances and stressors, like the physical and chemical stressors, to maintain its homeostatic state (Barton, 2002). Exposure of embryos to Cu and Ag leads to lower blood pressure as a result of the vasodilation of blood vessels that occurred due to the release of nitric oxide. To maintain the normal blood flow, cardiac output is stimulated, which goes up to compensate and maintain the blood flow by increasing the heart rates. On the other hand, oxidative damage of the cardiac tissue inhibits the cardiac Na pump that leads to alteration in K^+Na^+ ratio, recognized by K^+ leak to the ECF and increased intracellular Na^+ that altered the cardiac myocyte depolarisation.

The presence of cysteine, histidine (C_2H_2) as a constituent of zinc finger protein plays a role in activation of gene expression. Among the several tinmans of nkx (*nkx2.5*, *nkx2.3*, *nkx2.6*, nk*x2.7*, and *nkx2.8*), only *nkx2.5* and *nkx2.7* initiate the myocardial differentiation and are responsible for cardiac morphogenesis (Chen and Fishman, 1996; Tu et al., 2009). *Nkx2.5* appears to have a marked effect in the early stage of embryogenesis, and has a capability to initiate cardiogenic differentiation leading to the formation of the heart, especially at the early stage of gastrulation. In the current experiment, the *nkx2.5* gene showed increased expression as the time of the embryonic development progressed and reached the maximum expression at 16 hpf (segmentation stage) (Chapter 3). The level

of *nkx2.5* is on a gradient that initiates the ventral-marginal cells to be developed to form heart (Chen and Fishman, 1996). The interaction between the –SH of cysteine, histidine (C_2H_2) and Ag may impair the *nkx2.5* expression in 24 h aged embryos, as seen in the current study during Ag exposure (Chapter 4). Increased embryonic Cu accumulation did not affect *nkx2.5* expression. It is possible that the increased Cu accumulation met the requirement of the cardiac muscles to maintain the normal biochemical function particularly during the organogenesis of the heart. Normally, the high levels of GSH in the cardiac cells acts as a transporter of Cu via the formation of Cu-GSH complex to meet the requirement of cardiac muscles to form more Cu^{2+}/Zn SOD as an antioxidant, which may explain the failure of Cu to affect the expression of *nkx2.5* gene.

Increased *nkx2.5* expression was seen in embryos exposed to copper with added calcium. *Nkx2.5* expression increased as embryonic Cu accumulation increased and was concomitant with increased entrance of Ca into the embryo to compete for the increased Cu concentration. Thus, the increase in *nkx2.5* expression seems to be partly due to the role of calcium to activate the expression of the gene via the activation of RNA-binding protein (Chapter 3).

6.5. Proteomics as a tool to identified metal toxicity

Ecotoxicoproteomics represents a link between ecotoxicology and proteomics. As proteomics assay measured the type and concentration of the protein, it is also considered as being complementary to the biochemical assays. To my knowledge, the current study represents the first study that highlights the effect of silver on protein expression in zebrafish ELS at segmentation stage (24 hpf). In mass spectroscopy (MS) analysis, there were 810 types of proteins expressed in embryos exposed to silver concentrations at segmentation stage (24 hpf). Proteomics assay provides evidence that the exposure of embryos to Ag concentrations may affect the antioxidant enzymes. The previous experiment (Chapter 4) showed no effect of silver on total GSH level in 24 h live embryo, whereas the proteomics analysis revealed that silver exposure elicits

the production of glutathione S-transferase (GST), as an enzyme essential in the synthesis of GSH. In addition, proteomics showed that there is a possibility that Ag may cause the generation of OS. Increased fold of SOD (Cu-Zn) and CAT further to produce GST (not determined in the experiment-Chapter 4) is evidence that Ag may initiate the production of OS. On the other hand, the proteomics analysis showed an increase in ATP synthase subunit α and β, although the exposed embryos to Ag concentrations (Chapter 4) did not show significant effect on Na^+K^+-ATPase activity. The increase will provide evidence that there was stimulation for the synthesis of Na^+K^+-ATPase molecules to substitute the inactive enzyme and to meet the requirement of the developed osmoregulatory organs.

Among the expressed proteins, there were high numbers of uncharacterized proteins that interfere with my expectation to determine the presence of metallothionein and *nkx2.5*. More specific test such as western blotting test may be helpful to diagnose the specific proteins. However, the presence of high numbers of the expressed proteins represents the requirement of the developing embryos to maintain the formation and development of the organs during the organogenesis stage. Use of proteomics in ecotoxicology highlights the effect of pollutants with particular attention to the effect of trace metals; even the high concentrations of essential trace metals and the low concentrations of non-essential trace metals. Exposure of embryos to Ag concentrations for 24 h showed increased and decreased fold in expressed proteins. Among expressed proteins, some of the proteins like zona pellucida glycoproteins assist in the transfer of essential micronutrient trace elements such as copper and zinc and at the same time are considered as a route for the transfer of toxic metals such as silver and cadmium. The increase of stress proteins such as heat shock protein and chaperonin protect the embryo particularly at the early stage of the development when the embryos are more susceptible and sensitive to the toxic concentration of Cu and Ag, as shown in the current experiments (Chapter 3 and 4). Overall, the proteomics assay was a new protocol used to determine the type, behaviour (increased or decreased), and function of expressed proteins during the exposure of aquatic organisms particularly in their ELS.

Future work and recommendation

The aquatic environment is recognized by the presence of various types of pollutants, all acting together to affect the survival of the fish and other aquatic organisms at ELS of the development. It is well known that the intact embryos are more susceptible than dechorionated embryo to aquatic pollutants. Although the chorine acts as a barrier, the transition metals like Ag and Cu are easily accumulated in the PVF; through ion exchange or through the binding with sulfhydryl groups. Hence, according, the following ideas are recommended for future work:

- To assess the role of the chorion in the toxic effect of metals in the ELS, it is recommended to study the effect of Ag and Cu on osmoregulatory system, electrolytes balance, as well as the *nkx2.5* and *mt2* gene expressions in the dechorionated embryo.
- To assess the effect of Ag and Cu on the ELS, it is recommended to carry out the experiment on different stages of embryonic development by carrying out the dechorionation according to the different stages of development [blastula (2.25–5.25 h), gastrula (5.25–10.33 h), segmentation (10.33–24 h), hatched embryo (48–72 h)] and then expose the embryo to Ag and Cu. This will offer an idea about the behaviour of the chorion as a protective barrier.
- It is important to know the role of aquatic biotic ligands as competitive factors to reduce the toxic effect of Ag and Cu in dechorionated embryos. Exposure the embryos to Ca or humic acid before and during the exposure to Ag and Cu.
- Recently, engineered nanomaterials have been considered as sources of contamination of aquatic systems. In this context, it would be useful for risk assessment to assess the role of AgNPs and CuNPs as a source of Ag and Cu ions, and their effects on the early zebrafish life stage.
- More studies are needed to know the effect of Ag and Cu on the type, concentration, and the role of the proteins in the exposed embryo (intact and dechorionated embryo). This will involve refining the proteomics

analysis for applications in ecotoxicology as well as physiology. Other analyses that are used to detect specific proteins in the exposed embryo are also needed, including analytical techniques such as western blotting.

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Appendices

Appendix 1: Total Glutathione Assay

Reagent	Assay buffer 250 mL	Buffered 10 mmol L^{-1} DTNB 10 mL	2U mL^{-1} Glutathione Reductase 7 mL	3.63 mmol NADPH 2 mL
[100 mmol L^{-1}] potassium phosphate [KH_2PO_4]	3.402 g			
[5 mmol/l] Potassium EDTA	0.505 g			
MilliQ water	Make up to 250 mL			
2 M KOH	A few drops to adjust pH to 7.5			
DTNB Ellman's reagent - 5,5'-dithiobis-(2-nitrobenzoic acid)		0.0396 g		
NADPH *** *β*-Nicotinamide adenine dinucleotide phosphate tetrasodium salt				0.001774 g
Glutathione Reductase			9.9 µl + 6.99* mL assay buffer	

* Glutathione reductase calculation

2 mg protein mL^{-1} × 250U mg^{-1} = 500 U mL^{-1}

(0.71 U mL^{-1} / 500 U mL^{-1}) × 7 mL = 0.00994 mL × 1000 = 9.94 µl.

Assay Procedure

Note; this protocol is optimised for rainbow trout gill and liver tissue samples. You may need to carry out optimisation trials if using different animals/tissues etc. (e.g., volume of homogenate required).

Prepare samples, standards and blanks in triplicate. Note; assess the most convenient way to set up the microplate as this is a kinetic assay and as such time dependent when you start the reaction (initially run six samples in triplicate using three columns on the plate plus your blanks and standards).

1. Add 20 µl of buffered 10 mmol/l DTNB to each well.
2. Prepare the blanks in triplicate by adding 20 µl of distilled water to the appropriate wells.
3. Add 20 µl of each GSH standard (in triplicate) to appropriate wells.
4. Add 20 µl of homogenate to appropriate wells. You can increase this if required, but note that there needs to be a 1:1 ratio with the buffered DTNB.
5. Add 20 µl of 2 U/ml glutathione reductase to each well.
6. Add 260 µl assay buffer to each well.
7. After equilibration for 1 minute, start the reaction by adding 20 µl of 3.63 mmol/l NADPH from a pipetting reservoir with a multichannel pipettor. This is time critical in a kinetic assay.
8. Absorbance's can then be read at 412 nm for 15 minutes. This can be continuous measurements. The rate of change should be (e.g., every 30-60 seconds) after the reaction. The rate of change should be linearly related to the total GSH concentration.
9. Samples are expressed per gram wet weight tissue.

Appendix II: Determination of Na^+K^+-ATPase in Fish Tissues

Prepare the following ice-cold isotonic buffer:-
300 mmol L^{-1} Sucrose
0.1 mmol L^{-1} EDTA (ethylenedinitrilotetraacetic acid)
20 mmol L^{-1} HEPES (4-(2-hydroxyethyl)-1-piperazineerganesulfonic acid)
Adjust to pH 7.8 with Tris (2-amino-2-hydroxylmethyl-1,3-propanediol)

Reagent preparation for Na^+K^+-ATPase assay

Reagents	K^+ containing media (1 L)	K^+ free media (1L)
Sodium Chloride, 100 mmol L^{-1}	5.84 g	5.84 g
Potassium Chloride (99.0-100.5 %), 10 mmol L^{-1}	0.75 g	
Magnesium Chloride hexahydrate (min. 99%), 5 mmol L^{-1}	1.1 g	1.1 g
Na_2ATP, 1.25 mmol L^{-1}	0.689 g	0.689 g
Hepes buffer, 30 mmol L^{-1}, pH 7.4	7.149 g	7.149 g
Ouabain 1 mmol L^{-1}		0.729 g

Colour reagent

Reagents	Weight (g)
ammonium molybdate tetrahydrate (1.15% w/v)	1.15 % w/v
Iron(II) sulfate heptahydrate (⩾ 99.0%)	9.6 % w/v
H_2SO_4	0.66 M

To stop reaction: 8.6 % w/v Trichloroacetic acid (TCA)

Trichloroacetic acid (TCA): 0.68 g

MilliQ water : 100 mL

Assay procedure

1. Prepare blanks by adding 400 µl of either the K^+ containing or K^+ free (ouabain) media to test tubes in triplicate (depending on assay phase: K^+ or K^+ free).
2. Prepare standards by adding 400 µl of each standard (in triplicate) to clean test tubes. Typically for fish samples, phosphate standards of 0, 0.25, 0.5, 1.0 and 2.0 mmol/l made by dilution of 100 ml of 20 mmol/l potassium phosphate stock solution is linear.
3. Add about 15 µl of the homogenate (a total of 200-400 ng protein/sample) to 400 µl of each of the assay mediums on ice. Prepare in triplicate for each sample (gives 6 tubes per sample).
4. Incubate at 37 °C for 10 minutes (e.g. a water bath heated to 37 °C)
5. Stop the reaction by adding 1 ml of ice cold trichloroacetic acid (TCA 8.6% w/v) to all tubes (including blanks, standards and samples).
6. Read free phosphate by adding 1 ml of freshly prepared colour reagent (again to all tubes) by a change in absorbance at about 700 nm (Silva et al. used 660 nm). The 630 nm filter on the Dynex MRX plate reader works well. The colour development is usually complete in about 20 mins at room temperature.
7. Calibrate the colour development against the phosphate standards (this may need adjusting for particular tissues).

The Effect of Calcium on Accumulation and Toxicity of Dissolved Copper in the Early Stages of Zebrafish Embryo Development: Biochemical Effects and Gene Expression

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RESEARCH WITH PLYMOUTH UNIVERSITY

Introduction

- Copper is an essential trace metal required by living organisms. Fish larvae and early juveniles are sensitive to copper at low µg l^{-1} concentrations. Unhatched embryos can also be affected by copper toxicity. The early life stages (ELS) of zebrafish are well known to be sensitive to low concentrations of dissolved metals, especially before the hardening of the egg chorion.
- Fishes can be protected from copper toxicity. Calcium is one of the main cations of water hardness and thought to limit the copper toxicity due to its competition with copper ions for the anionic binding sites on the gill. In addition, metallothionein induction is well known in aquatic organisms during trace metal exposure, and acts to chelate the excess intracellular metal ions to prevent their toxicity.
- Sodium homeostasis is a target for copper toxicity in fishes. The Na^+ pump is vulnerable to Cu-dependent inhibition, and interference with other sodium transporters or channels may result in whole-body ionoregulatory disturbance in adult fish (1). However, in excitable tissues like the heart, sodium homeostasis is also linked to calcium via the Na^+/Ca^{2+} exchanger. The heart is the first organ to form and function in the vertebrate embryos, and the effects of Cu exposure on cardiac function are poorly understood. The Na^+ pump with the Na^+/Ca^{2+} exchangers are essential to regulate cardiac functions, including contractility and depolarization of the muscle (2).

Aims

- The overall aim was to investigate whether or not the toxicity of copper in zebrafish embryos was affected by added calcium to the external water, and the consequences for ionic regulation and cardiac development.

Experimental Design

- Twenty live and dead embryos were collected at 16 hpf for ICP-MS analysis to estimate the embryonic concentrations of Ca^{2+}, Cu, K^+, and Na^+.
- Sixty live embryos were collected at 16 hpf to determine the Na^+K^+-ATPase activity as osmotic/ionic regulator and total glutathione as carrier and detoxifying factors were evaluated as biochemical markers to investigate the toxicity of copper in zebrafish embryos.
- One hundred and twenty live embryos were collected at 16 hpf to investigate the effect of copper on the expression of nkx 2.5 as one of the genes which are responsible for the heart formation and development in embryos, and Mt2 gene expressions as an indicator of trace metals exposure and detoxifying factor by using the (qRT-PCR).

Results

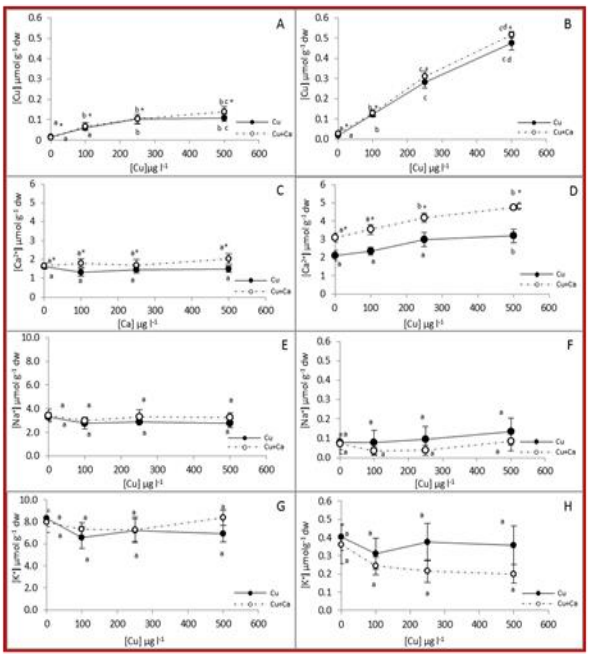

Fig.1. Metal concentrations in live embryos (A, C, E, G) and dead embryos (B, D, F, H). Samples were collectedafter16h of exposure to copper without added calcium (black circles, black unbroken line) and with added calcium (open circles, dotted lines). Data are means ± S.E.M., n = 6 samples per treatment. Different letters indicate a significant effect (ANOVA, P < 0.05). Same letters within treatment indicate no significant effect (ANOVA, P>0.05). * Indicate a significant effect of calcium between with and without added calcium groups (ANOVA, P<0.05).

Acknowledgment

This study is supported by Iraqi Ministry of Higher Education & Scientific Research.

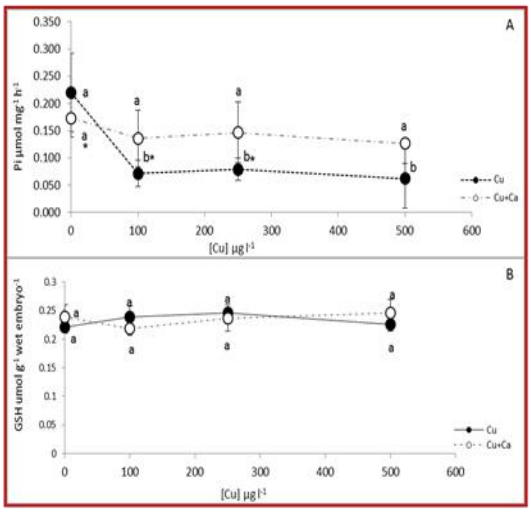

Fig.2. Na^+K^+-ATPase activity (A), total glutathione (B) in zebrafish embryos following copper exposure with and without added calcium. Copper (black circles, black fine dotted lines) and copper with added calcium (open circles, light dotted lines). Data are means ± S.E.M, n= 6 samples per treatment. Different letters indicate a significant effect within the group (ANOVA, $p < 0.05$). The same letters indicate no significant effect within the group (ANOVA, $p > 0.05$). * Indicate a significant effect of calcium between with and without added calcium groups (ANOVA, $p < 0.05$).

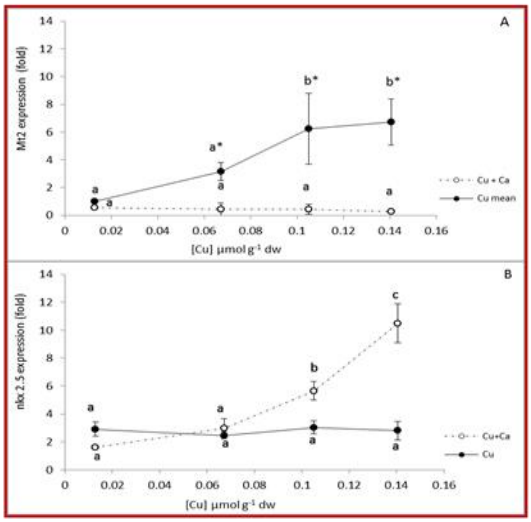

Fig.3. Expression of Mt2 (A) and nkx 2.5 (B) linking to Cu^{2+} accumulation in live embryos exposed to Cu only (black circles, unbroken black line) and with added Ca (open circles, dotted lines). Embryos were sampled at 16 hpf. Fold changes in gene expression were calculated by the $2^{\Delta\Delta CT}$ method with β-actin used as a housekeeping gene. Data are means ± S.E.M., n = 3 samples per treatment. Different letters indicate a significant effect (ANOVA, P < 0.05). Same letters within treatment indicate no significant effect (ANOVA, P>0.05). * Indicate a significant effect of calcium between with and without added calcium groups (ANOVA, P<0.05).

Conclusion

- Impairment of osmoregulation by copper is pronounced in zebrafish embryos.
- The interaction between added Ca, Cu exposure and osmoregulation added further insights on the ameliorative effect of Ca during copper exposure.
- Increased levels of Mt mRNA following exposure to copper presumably due to copper induced oxidative stress, suggesting Mt plays a role in detoxifying copper.
- The expression of nkx 2.5 genes was not affected by Cu exposure alone, but becomes sensitive to Cu in the presence of added Ca.
- However, further work on the interaction of Cu and Ca on nkx 2.5 gene expression in the heart is needed.

References

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(2) Shu, X., Cheng, K., Patel, N., Chen, F., Joseph, E., Tsai, H.-J., Chen, J.-N., (2003) Na,K-ATPase is essential for embryonic heart development in the zebrafish. Development 130, 6165-6173

The effect of waterborne silver on survival, ionoregulatory function, metallothionein and *nkx2.5* inductions in zebrafish (*Danio rerio*) embryos

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Introduction

❖Natural leaching of silver and anthropogenic activities that include use of silver nanoparticles Ag-NPs that are used in products to impart antimicrobial properties, lead to elevation of silver concentrations in the aquatic environment.

❖Silver salts such as $AgNO_3$ are soluble and dissociates to yield free silver ions (Ag^+). The ionic form of silver (Ag^+) is extremely toxic to the aquatic organisms [1].

❖Early life stage of fish show greater sensitivity to silver toxicity and effects include delayed hatching and decreased survival.

❖The impairment of Na^+K^+-ATPase activity appears to be key mechanisms of toxicity of silver and other toxic metals [2].

❖However, the effects of silver on fish embryos and their development are less well understood.

Aims

❖Investigate toxicity of waterborne silver on the survival, hatching and cardiac function in early life stage of zebrafish.

❖Evaluate whether the silver exposure during the early life stages is associated with impairment of the osmoregulatory system (Na^+K^+-ATPase and electrolyte levels) as is observed in adult fish.

❖Determine the role of silver on *mt2* induction and *nkx2.5* expression.

Experimental design

❖ Embryos (1 < hpf) were exposed in six replicate (n = 170 embryos per beaker) to concentrations 0, 2.5, 5, 7.5, 10, and 15 µg Ag^+ l^{-1} for 72 hpf.

❖ Embryos were collected as follows:

- For the estimation of embryonic metal concentration: Twenty live and dead embryos were collected at 24 hpf, and 10 live hatched embryos were collected at 72 hpf for ICP-MS analysis to estimate the embryonic concentrations of Ca^{2+}, Cu, K^+, and Na^+.
- For the biochemical assays: Fifty live embryos and 25 hatched live embryos were collected at 24 and 72 hpf respectively, to determine the Na^+K^+-ATPase activity as osmotic/ionic regulator and total glutathione as detoxifying factors were evaluated as biochemical markers to investigate the toxicity of silver in zebrafish embryos.
- For molecular assays: hundred live embryos and 30 live hatched embryos from each beaker were collected at 24 and 72 hpf respectively to investigate the effect of silver on the expression of *nkx2.5* as one of the genes which are responsible for the heart formation and development in embryos, and *mt2* gene expressions as an indicator of trace metal toxicity.

Result

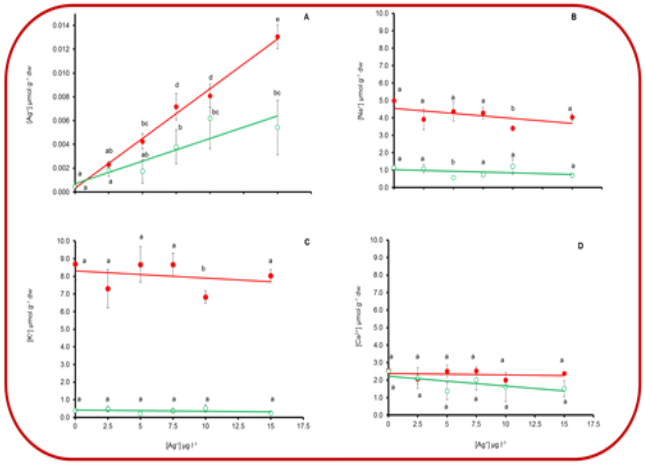

Figure 1. Metals concentrations [µmol metal g^{-1} dry weight of the embryo (±SE)] in live (solid circle), and dead (open circle) zebrafish embryos at 24 hpf relative to Ag^+ exposure concentrations. The concentrations of silver (A), sodium (B), potassium (C), and calcium (D) were assessed in embryos exposed to Ag^+. Each exposure condition had six independent replicates and each replicate contained 170 embryos at the beginning of the exposure. The number of live and dead embryos analyzed for each condition was (20). Significant relation between live and dead embryos at 24hpf (ANOVA, $p < 0.05$).

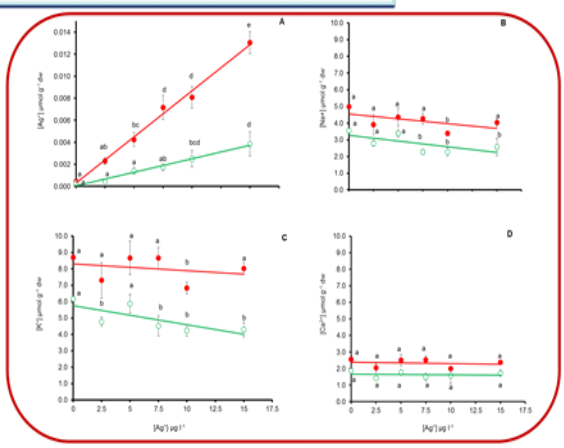

Figure 2. Metals concentrations [µmol metal g^{-1} dry weight of the embryo (±SE)] in live zebrafish embryos at 24 hpf (solid circle), and 72 hpf (open circle) relative to Ag^+ exposure concentrations. The concentrations of silver (A), sodium (B), potassium (C), and calcium (D) were assessed in embryos exposed to Ag^+. Each exposure condition had six independent replicates and each replicate contained 170 embryos at the beginning of the exposure. The number of live embryos analysed for each condition was (20). Significant relation between live 24 hpf and 72 hpf embryos (ANOVA, $p < 0.05$).

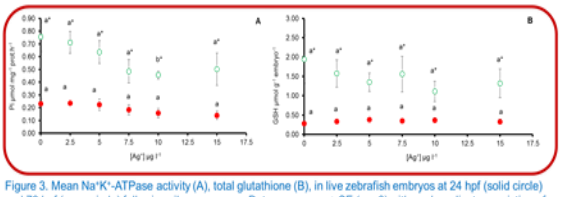

Figure 3. Mean Na^+K^+-ATPase activity (A), total glutathione (B), in live zebrafish embryos at 24 hpf (solid circle) and 72 hpf (open circle) following silver exposure. Data are means ± SE (n = 6) with each replicate consisting of 170 embryos. Different letters indicate the differences within the concentrations (ANOVA, $p < 0.05$).

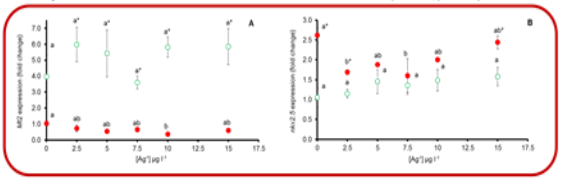

Figure 4. Expression of mt2 (A) and nkx2.5 (B) relative to silver concentrations. Embryos were sampled at 24 hpf (solid circle) and 72 hpf (open circle). Fold changes in gene expression were calculated by the 2ΔΔCt method with β-actin used as a housekeeping gene. Data are means ± S.E.M., n = 3 samples per treatment. Different letters indicate a significant effect within the concentrations (ANOVA, $P < 0.05$).

Conclusion

❖ At 24 hpf, accumulation of silver was higher in live embryos compared to dead embryos indicating that live embryos were able regulate accumulation of silver to some extent (Figure 1A). Live age 24 h embryos also had higher K^+ and Na^+ concentrations than dead embryos indicating that ion regulation was functioning (Figure 1 B and C).

❖ Accumulation of silver was greater in live zebrafish at age 24 h than at age 72 h indicating reduced ability of hatched embryos to manage exposure to aqueous silver (Figure 2A). Age 24 h embryos did have higher K^+ and Na^+ concentrations than age 72 hour embryos; however, this could be an indication of greater amounts of these ions in the yolk rather than enhanced ionoregulatory ability of 24 hour-old embryos (Figure 2 BC).

❖ The interaction between silver exposure and osmoregulation added further insights on the effect of silver on the early life stage (ELS) of zebrafish.

❖ Increased levels of *mt2* mRNA following exposure to silver presumably due to silver induced oxidative stress, suggesting metallothionein plays a role in detoxifying silver.

❖ The expression of *nkx2.5* genes was affected by silver exposure during the 24 hpf (early stage of embryonic development), whereas, not affected at 72 hpf (hatched embryos), which indicate the importance of *nkx2.5* gene for the formation and development of the heart during the early stage of embryonic development.

Acknowledgment

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